

**The 78 kDa glucose regulated protein
(GRP78) as a potential treatment
predictive biomarker and therapeutic
target in colorectal cancer adjuvant
chemotherapy**

Thesis submitted in accordance with the
requirements of the University of Liverpool
for the degree of Doctor in Medicine (MD)

By

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Declaration & Statement of Originality

This thesis is the result of my own work. The material contained in the thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or other qualification.

The research was carried out in the Division of Surgery and Oncology, School of Cancer Studies, University of Liverpool, between January 2010 and January 2012.

Abstract

Introduction: Glucose-regulated protein 78-kDa (GRP78) is an endoplasmic reticulum (ER)-resident molecular chaperone that is essential for correct protein folding and assembly in the ER lumen. Micro-environmental stress and a requirement for increased protein synthesis, typical of solid tumours, leads to a disruption of ER homeostasis, and accumulation of misfolded proteins. The ability of GRP78 to dissociate from several important ER-resident transmembrane proteins under conditions of ER stress leads to a cascade of signal transduction pathways, known as the unfolded protein response (UPR), that modulate cell survival or, if the stress is significantly severe, apoptosis. GRP78 has been found to be overexpressed in a variety of cancers compared with benign tissue and has been associated with poor outcome. In-vitro data indicate that GRP78 expression is often associated with aggressive phenotype and drug resistance. Thus, GRP78 has potential as a biomarker for tumour behaviour and treatment response.

For stage III colorectal cancer, there is overwhelming evidence to recommend the use of fluoropyrimidine-based adjuvant chemotherapy. Unfortunately, a large proportion of patients do not benefit from adjuvant chemotherapy, and biomarkers that can determine the likelihood of response to chemotherapy remain elusive. The benefit of chemotherapy in stage II disease is less certain and markers that could reliably predict benefit would be particularly useful in this population. This study explores a potential mechanistic relationship between GRP78 and 5-FU sensitivity using both siRNA transfection and treatment with an engineered fusion protein, epidermal growth factor (EGF)-SubA, which has been demonstrated to cause highly selective cleavage of GRP78 at a single amino acid point. It was then examined

whether GRP78 may have prognostic or predictive value in the context of colorectal cancer patients treated with fluoropyrimidine-based chemotherapy. The potential therapeutic value of targeting GRP78 in vitro using EGF-SubA is also examined.

Methods: Colon cancer cell lines were used to examine response to 5-FU upon modulation of endogenous GRP78 using siRNA technology and EGF-SubA. Apoptosis and cell cycle progression were assessed using flow cytometry. Immunohistochemistry was used to characterise GRP78 expression in a large cohort of colorectal cancers on tissue microarrays and the results were correlated with clinicopathological parameters and with 5-year survival for the whole cohort and those treated with fluoropyrimidine-based (5-FU) adjuvant chemotherapy. The action of EGF-SubA upon colon cancer cells was examined using western blotting, MTT assay and flow cytometry.

Results: GRP78 promotes apoptosis in response to 5-FU. Better overall 5-year survival was associated with high GRP78 expression ($P=0.036$). Stage III patients with high GRP78 showed significant benefit from adjuvant chemotherapy ($P=0.026$), whereas patients with low GRP78 failed to benefit ($P=0.805$). Low GRP78 was an independent poor prognostic indicator of overall 5-year survival ($P=0.005$; $HR=1.536$; 95%CI 1.139-2.122). Colon cancer cells expressing EGFR were highly sensitive to EGF-SubA, demonstrating reduced proliferation and cell cycle arrest. However, EGF-SubA did not induce significant apoptosis and reduced the effectiveness of 5-FU in vitro.

Conclusion: This study demonstrates a mechanistic relationship between GRP78 expression and response to 5-FU. GRP78 expression may provide a useful additional risk stratification to inform the adjuvant treatment of colorectal cancer. EGF-SubA does not have therapeutic value in colorectal cancer but is a useful tool for studying GRP78 and the UPR.

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Prizes/Awards, Presentations relating to the work herein

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3. ***ACPGBI/RSM Travelling Fellowship*** entitled ‘GRP78 in colorectal cancer as a prognostic indicator and novel therapeutic target’ presented at the Colorectal Tripartite Meeting, Cairns, Australia, July 2011.

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A copy of this paper is enclosed in the Appendix section 7 of this thesis (Page 206).

Presentations

International Conferences

1. ***Poster presentation*** entitled ‘GRP78: a biomarker for and determinant of response to adjuvant chemotherapy in colorectal cancer’ at the European Colorectal Congress, St. Gallen, Switzerland, November 2011.
2. ***Oral presentation*** entitled ‘GRP78 in colorectal cancer as a prognostic indicator and novel therapeutic target’ presented at the Colorectal Tripartite Meeting, Cairns, Australia, July 2011.

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3. ***Poster presentation*** entitled ‘Investigating GRP78 as a potential therapeutic target in colon cancer using a novel engineered fusion protein; EGF-SubA’ at the National Cancer Research Institute Cancer Conference, BT Convention Centre, Liverpool, November 2011.

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Regional/Local Meetings

1. **Oral presentation** entitled ‘GRP78: a biomarker for and determinant of response to adjuvant chemotherapy in colorectal cancer’ at the Annual Meeting of the Liverpool and Northwest Society of Surgeons, The Liverpool Maritime Museum, December 2011.
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List of abbreviations

ABC	Avidin biotin complexes
ACF	Aberrant crypt foci
ADH	Alcohol dehydrogenase
AJCC	American Joint Committee on Cancer
APC	Adenomatous polyposis coli
APS	Ammonium persulphate
ASR	Age standardised ratio
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
Bax	Bcl-2-associated X protein
Bcl-2	B-cell leukaemia/lymphoma 2
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CDK	Cyclin dependent kinase
CHOP	C/EBP homologous protein
CI	Combination index
CRC	Colorectal cancer
CRM	Circumferential resection margin
CRT	Long course chemoradiotherapy
CT	Computed tomography
DAB	3,3'-diaminobenzidine
DCC	Deleted in colorectal cancer

DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxy-ribonucleic acid
DPD	Dihydropyrimidine dehydrogenase
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eIF2 α	α subunit of eukaryotic translation initiation factor
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
FA	Folinic acid
FAP	Familial adenomatous polyposis
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
5-FU	5-Fluorouracil
GRP78	Glucose regulated protein 78kDa
Gy	Gray
HIF	Hypoxia inducible factor
HNPCC	Hereditary non-polyposis colorectal cancer
HRP	Horse radish peroxidase
HSF	Heat shock factor
HSP	Heat shock protein
IC	Inhibitory concentration
IHC	Immunohistochemistry
IRE-1	Inositol-requiring enzyme 1 alpha

MEF	Mouse embryonic fibroblast
MRI	Magnetic resonance imaging
MSI	Microsatellite instability
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NEAA	Non essential amino acids
NICE	National Institute of Clinical Excellence
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PBST	PBS Tween
PCR	Polymerase chain reaction
PERK	Double strand RNA-activated protein kinase-like ER kinase
PET	Positron emission tomography
PI	Propidium iodide
PI3K	Phosphatidylinositol 3 kinase
PMSF	Phenyl methane sulfonyl fluoride
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCPRT	Short course preoperative radiotherapy
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
siRNA	Small interfering RNA
TCA	Tricarboxylic acid cycle
TMA	Tissue micro array
TME	Total mesorectal excision

TNM	Tumour, node, metastasis
UICC	International Union Against Cancer
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
WT	Wild-type
XBP1	X-box-binding protein

1 Introduction

1.1 Colorectal Cancer

1.1.1 Demographics

In the 21st century, colorectal cancer remains an important disease causing significant morbidity and mortality. Colorectal cancer is the third most common cause of cancer related death (after breast and lung cancer) in the United Kingdom and the second commonest in non smokers. Over 100 new cases of colorectal cancer are diagnosed each day in the UK. Around 40,000 new cases of colorectal cancer (2/3 in the colon and 1/3 in the rectum) are registered in the United Kingdom each year and approximately 16,000 deaths,(1-4) but there has been a substantial improvement in five year survival, from 22% to 50% over the last 30 years.(5, 6) Worldwide, there are approximately 1.2 million cases of colorectal cancer and over 600,000 people die each year.(7) The incidence of colorectal cancer rises with age with 84% of cases arising in people who are 60 years or older (Figure 1.1.1-1).

Rates are similar for males and females until age 50 and thereafter males predominate. The lifetime risk of being diagnosed with colorectal cancer in the UK is estimated to be 1 in 14 for men and 1 in 19 for women.(8)

In Europe in 2008, colorectal cancer was the most common cancer with 436,000 new cases and the second most common cause of cancer death with 212,000 deaths. The incidence of colorectal cancer has increased modestly in most European countries, especially in men, although mortality rates have in general declined.(9)

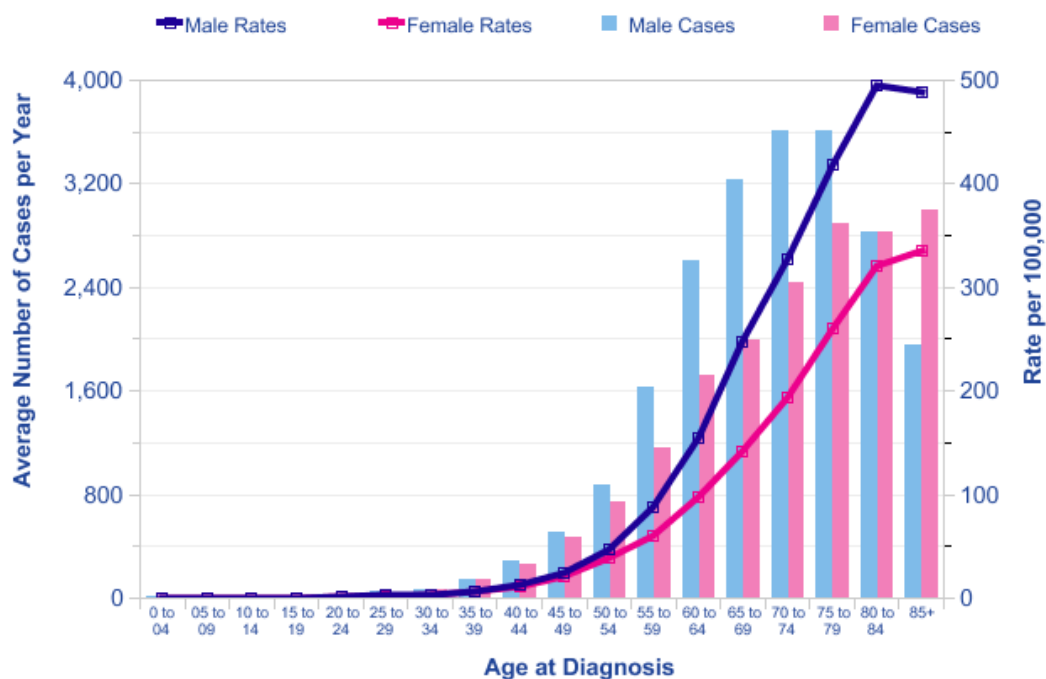


Figure 1.1.1-1: Age-specific incidence of colorectal cancer in the UK in 2011, for men and women.(8)

There is a wide geographical variation in distribution of colorectal cancer globally with almost 60% of cases occurring in the more affluent developed countries such as North America, Australia/New Zealand, Western Europe and Japan, with lower incidence seen in Africa, Asia and South America.(10) This large geographical variation is probably due to environmental exposures.

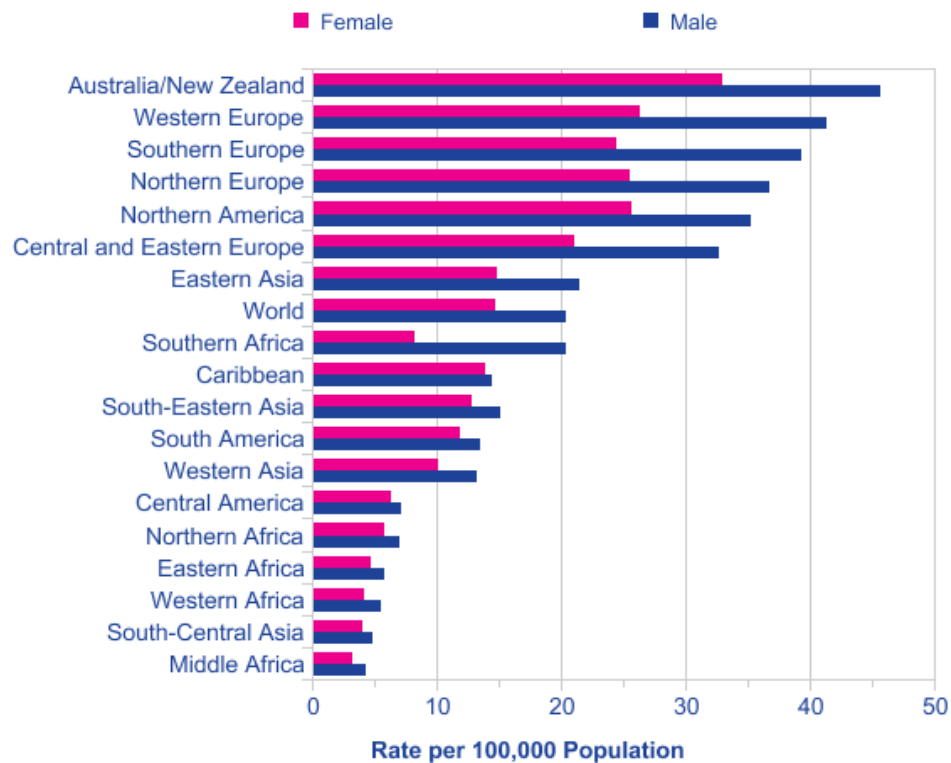


Figure 1.1.1-2: Geographical distribution of colorectal cancer in 2011.(8)

1.1.2 Molecular basis of colorectal cancer

For decades, our understanding of colonic carcinogenesis has been underpinned by the concept of the ‘adenoma-carcinoma’ sequence, as first discussed in 1951 by Jackmann and Mayo.(11) Evidence supporting the adenoma-carcinoma hypothesis is based on strong but circumstantial observations. Supporting evidence includes:

- The prevalence of adenomas is very similar to that of carcinomas and the average age of adenoma patients is approximately 5 years younger than carcinoma patients.(12)
- Remnants of adenoma are often found in resected colon cancer, particularly adjoining small early cancers.(13)

-
- Familial adenomatous polyposis (FAP) is unequivocally pre-malignant and the adenomas resulting from this condition are identical to sporadic adenomas.(14, 15)
 - Cellular atypia and gene mutations are more common in large adenomas than small. Adenomas greater than 2cm have a 50% chance of containing foci of invasive cancer.(16-18)
 - The distribution of adenoma throughout the colon is almost identical to that of carcinomas.(17-19)
 - Synchronous adenomas may be found in a third of colorectal cancer surgical resections.(20, 21)
 - Endoscopic removal of adenomatous polyps reduces the long term risk of colorectal cancer.(22, 23)

It was felt however that this simple hypothesis could not account for all cases of colon cancer. Contemporary genetic analysis has subsequently expanded our knowledge of this pathway.

1.1.2.1 The chromosomal instability pathway

The seminal paper by Vogelstein et al.,(24) described the accumulation of genetic mutations leading to the formation of a benign adenoma and subsequent transformation to invasive malignancy. The chromosomal instability pathway identifies inactivating mutations of the adenomatous polyposis coli (APC) tumour suppressor gene on chromosome 5q21, implicated in cell to cell adhesion, is found in 60% of all adenomas and carcinomas, and probably occurs early.(25) Activating mutations of the KRAS oncogene, responsible for activating growth factor signal

transduction, is also a common finding,(26) but more so in larger adenomas and may represent a later event.(24) A deletion on chromosome 18q is seen in 70% of carcinomas and in almost 50% of late adenomas. The region lost includes the deleted in colorectal cancer (DCC) tumour suppressor gene, which encodes proteins involved in cell adhesion. The DCC gene is expressed in normal mucosa but expression is reduced or absent in the majority of colorectal carcinomas.(27) The loss of a large portion of chromosome 17p, containing the p53 tumour suppressor gene, is seen in more than 75% of colorectal carcinomas, but is rare in adenomas at any stage.(24, 28) p53 mutation is therefore thought to represent a late event in the transition from adenoma to high-grade dysplasia and lays the stage for malignant transformation. A suggested model of the series of genetic events involved in the adenoma-carcinoma sequence is shown below.

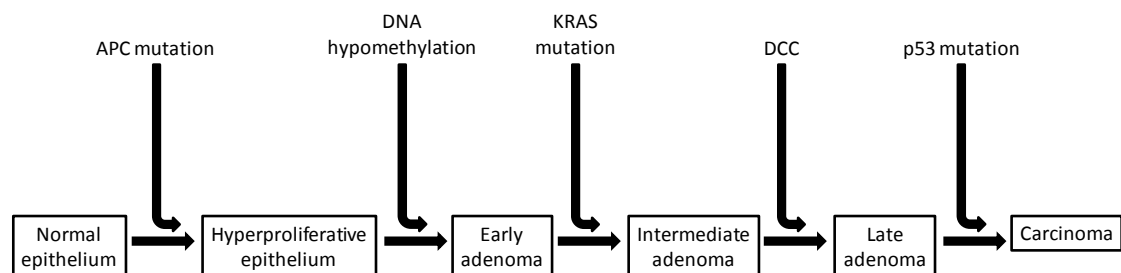


Figure 1.1.2-1: Proposed adenoma-carcinoma sequence in colorectal cancer.(29)

Many other genetic abnormalities are described in colorectal cancer but there is no single mutation common to all cancers. The sequence proposed above represents one possible scenario.

Germline mutation of the APC gene leads to familial adenomatous polyposis (FAP). FAP is a dominantly inherited condition characterised by the appearance of multiple adenomatous polyps throughout the colon which inevitably lead to colorectal cancer by the fourth decade of life. The severity of polyposis varies depending on the

specific mutation, with mutation at codon 1309 (exon 15) being associated with a very dense polyposis. FAP accounts for 1% of all colorectal cancers. Patients require a colectomy at the age when polyps start to develop, usually in late teenage years and early 20s.(30)

1.1.2.2 Microsatellite instability pathway and Lynch syndrome

Whilst 85% of sporadic colorectal cancers display chromosomal instability, the remaining 15% demonstrate a microsatellite instability (MSI) phenotype.(31) Whilst sporadic cancers caused by MSI are usually attributable to somatic hypermethylation of the promoter region of MLH1 gene, up to 4% of colorectal cancers are caused by Lynch syndrome, an autosomal-dominant cancer predisposition characterised by insertion-deletion germline mutations of short tandemly repeated nucleotides (microsatellites) caused by defects in DNA mismatch repair (MMR) genes (MLH1, MSH2, MSH6, PMS1 and PMS2). Chromosome losses are rarer in these tumours and in contrast to the aneuploidy or polyploidy karyotypes seen in tumours displaying chromosomal instability, MSI tumours are typically diploid.(32) Histologically, tumours with MSI differ from typical CIN cancers in that they are often proximally located, poorly differentiated and of 'signet ring' appearance, mucinous, and show marked lymphocytic infiltration.(33)

Lynch syndrome (previously known as hereditary non-polyposis colorectal cancer or HNPCC) is the most common hereditary colon cancer syndrome and is associated with a lifetime risk of colorectal cancer of around 80%, plus an increased risk of a variety of other cancers (endometrial, ovarian, gastric, small bowel, and ureter).(34)

Before wide availability of genetic testing, those at risk of Lynch syndrome were identified on the basis of clinical and family history criteria (Amsterdam II or revised Bethesda criteria).

Table 1.1-1: Amsterdam II criteria (35)

Amsterdam II criteria for gene testing for Lynch syndrome

At least 3 relatives with a Lynch-associated cancer (colorectal, endometrial, small bowel, ureter, renal pelvis)

One affected family member is a first-degree relative of the other two

At least two successive generations affected

At least one diagnosed before age 50 years

Familial adenomatous polyposis has been excluded

Tumours are verified by pathological examination

Table 1.1-2: Modified Bethesda guidelines (36)

Modified Bethesda guidelines for testing colorectal tumours for microsatellite instability

Diagnosed with colorectal cancer before the age of 50 years

Synchronous or metachronous colorectal or other HNPCC-related tumours (which include stomach, bladder, ureter, renal pelvis, biliary tract, brain (glioblastoma), sebaceous gland adenomas, keratoacanthomas and carcinoma of the small bowel), regardless of age

Colorectal cancer with a high-microsatellite instability morphology that was diagnosed before the age of 60 years

Colorectal cancer with one or more first-degree relatives with colorectal cancer or other HNPCC-related tumours. One of the cancers must have been diagnosed before the age of 50 years (this includes adenoma, which must have been diagnosed before the age of 40 years)

Colorectal cancer with two or more relatives with colorectal cancer or other HNPCC-related tumours, regardless of age

The Amsterdam criteria were the first diagnostic guidelines to be developed with the aim of determining whether a family should be classified as having HNPCC. The Bethesda guidelines were developed with a different objective to the Amsterdam criteria and are useful in deciding whether individuals with cancer in families that do not fulfil Amsterdam criteria should undergo genetic testing (Figure 1.1.2-2).(37)

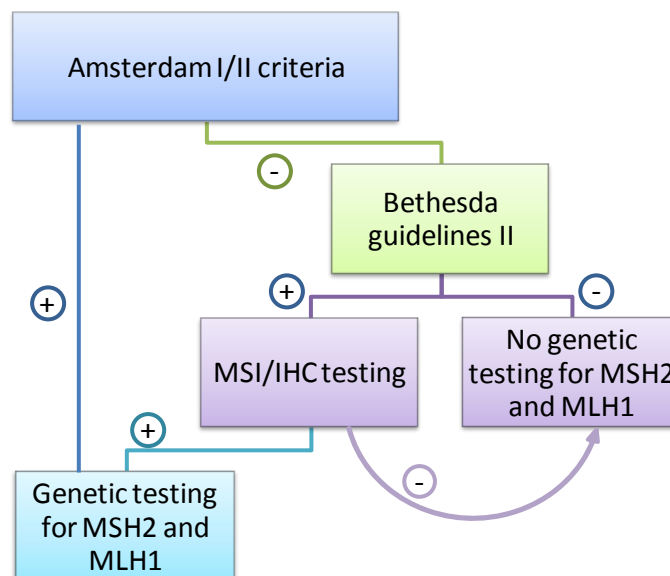


Figure 1.1.2-2: Strategy for genetic testing of affected individuals from families with suspected hereditary non-polyposis colorectal cancer.(37)

To limit the expense of performing full germline mutational analysis of all candidate patients, preselection is performed based on biomarkers. Using PCR, MSI can be detected by analysis of a panel of 5-10 microsatellite sequences in the DNA of a tumour compared with the DNA of normal adjacent mucosa.(37) Tumours fall into one of three categories: MSI-High (MSI-H), unstable for 30% of markers used; MSI-Low (MSI-L), unstable for 10-30% of markers; or microsatellite stable (MSS), for cases with no MSI. Immunohistochemistry for lack of expression of MMR proteins (primarily MLH1 protein) is also diagnostic for defective mismatch repair and can be used as an alternative to PCR.(38) Although these techniques can determine the

presence of mismatch repair deficiency, they are not able to distinguish Lynch syndrome cancers from sporadic MSI cancers. It is now known that BRAF mutation is associated with MLH1 promoter region hypermethylation and not germline mutations in the mismatch repair (MMR) genes.(39) Therefore it is possible to distinguish between sporadic MSI tumours and those due to Lynch syndrome using a simple algorithm (Figure 1.1.2-3).

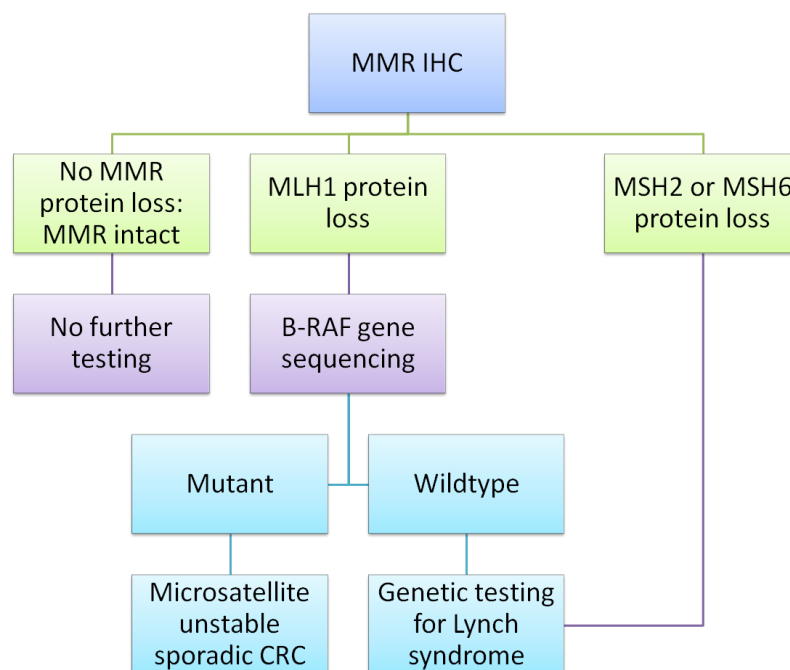


Figure 1.1.2-3: Algorithm for genetic testing for Lynch syndrome. Modified from (40)

1.1.2.3 The epigenetic pathway to colorectal cancer

Non-Lynch MSI tumours are similar to Lynch-associated colorectal cancers histologically and in that they are more likely to occur in the right colon and have a better overall prognosis.(41) However, these sporadic MSI tumours do not demonstrate a strong inheritance pattern and do not occur at a young age. Unlike Lynch syndrome, gene mutations are not present and the MSI is known to be due to hypermethylation of the promoter region of MLH1 leading to its inactivation. This

overlaps with the observation that some colonic polyps and cancers are characterised by hypermethylation of a large number of genes, a process termed the CpG (Cytosine-phosphate-Guanine) island methylator phenotype (CIMP).(42) CpG island hypermethylation is a critical mechanism for tumour-suppressor gene silencing.(43)

1.1.3 Pathology

Among the earliest premalignant lesions in colorectal cancer are aberrant crypt foci. Dysplastic aberrant crypt foci (ACF), also referred to as adenomatous crypts or micro adenoma, frequently show loss of heterozygosity in the APC gene. ACF are widely believed to be precursors to adenomatous polyps. In normal tissue, colonic stem cells at the bottom of crypts divide continually in an asymmetric manner, feeding cells into the proliferative zone of the crypt. This preserves the stem cell and gives rise to a daughter committed to differentiation and death. Following APC gene mutation, an initiated stem cell may lose the ability to divide asymmetrically and occasionally divides symmetrically giving rise to two initiated daughter cells which retain the ability of clonal expansion. As these cells divide and move from the stem cell zone to the proliferative zone of the crypt, they become freed from the microenvironmental constraints of the crypt and are free to expand clonally via symmetric divisions. If not compensated for by apoptosis, altered stem cells may accumulate. A further genetic event, such as a mutation of one copy of the p53 gene acting in a dominant negative manner, may be the catalyst for subsequent malignant transformation of these initiated stem cells.(44)

Adenomas are present in around 30% of the population,(45) and can be classified histologically as tubular, tubulo-villous, or villous. Dysplastic change may be classed

as low grade or high grade based on the degree of architectural abnormality and cellular atypia. More than 90% of adenomas are small (< 1 cm in diameter) and do not progress. Risk factors for malignant progression include the presence of multiple adenomas, size greater than or equal to 1 cm, and villous histology or severe dysplasia in adenomas of any size.

Colorectal cancers are typically described as polypoidal, ulcerating, annular or mucinous. The intraluminal part of tumour may be large, filling the entire lumen, so as to cause obstruction. The vast majority of cases of colorectal cancer are adenocarcinoma and are commonly subdivided into well, moderate or poorly differentiated depending on the degree of cellular atypia, mitotic rate and glandular architecture. Generally, well differentiated tumours have a better prognosis and poorly differentiated the worst prognosis but in practise most tumours are moderately differentiated.

The commonest tumour type is adenocarcinoma. Most are moderately differentiated and tend to demonstrate cribriform patterns with central necrosis. Mucinous adenocarcinoma is a subtype that secretes extracellular mucin, and may be associated with microsatellite instability. Medullary carcinoma is an important subtype of colorectal cancer with a specific phenotype – right sided tumours with sheets of cells and numerous tumour-infiltrating lymphocytes on microscopy. Medullary carcinoma is associated with HNPCC. Less common tumours include signet ring cell carcinoma, with distinctive intracytoplasmic mucin; and small cell carcinoma, a poorly differentiated neuroendocrine carcinoma.(46)

The majority of colorectal cancers occur in the sigmoid colon or rectum. The descending and transverse colon are involved infrequently with the ascending colon and caecum affected more often (Figure 1.1.3-1).

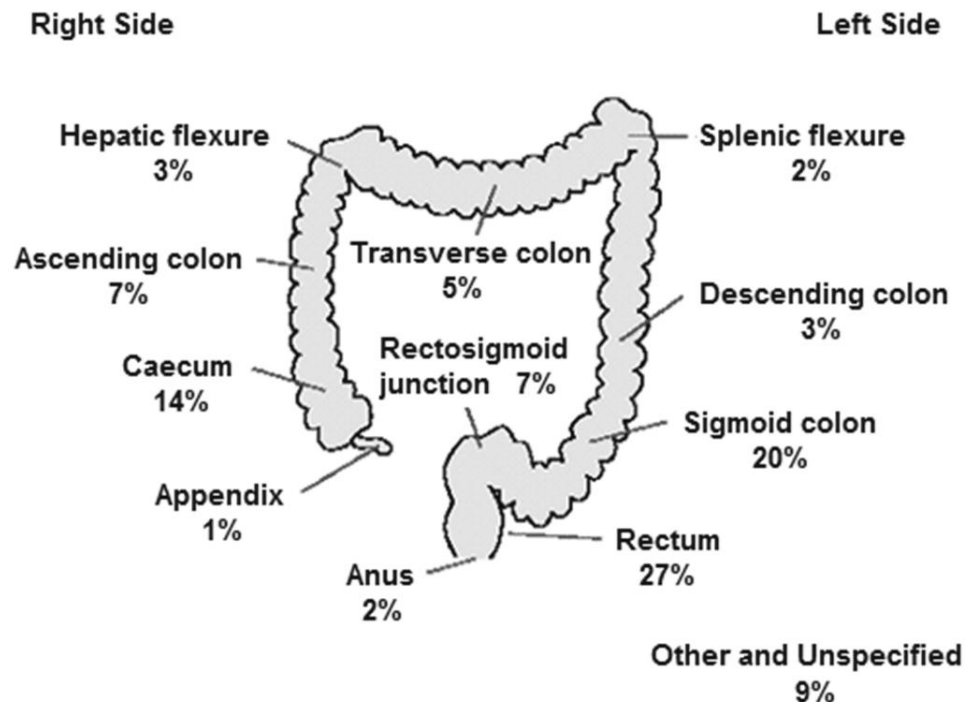


Figure 1.1.3-1: Distribution of colorectal cancers.(8)

1.1.3.1 Histopathological staging

The purpose of staging is to allow the clinician to advise the patient as to prognosis and as a guide to the most appropriate form of treatment, as well as allowing a meaningful comparison of the outcomes of treatment between similar groups of patients. The most common staging systems are Dukes and the TNM system.

1.1.3.2 Dukes system

In 1932, the British pathologist Cuthbert Dukes published his classification system for colorectal cancer.(47) It is based on the depth of invasion of the cancer through the bowel wall and lymph node involvement and was originally described for rectal

resections but is extrapolated to colonic resections. The system has subsequently been modified somewhat but still retains the basic ABC classification:

- Stage A, tumour confined to the bowel wall (including muscularis mucosa)
- Stage B, tumour penetrating the muscularis mucosa
- Stage C₁, regional lymph nodes involved by tumour but not affecting the node closest to point of ligature
- Stage C₂, lymph node at the point of ligature involved by tumour
- Stage D, distant metastasis (not part of Duke's original classification)

1.1.3.3 TNM Staging

The tumour, node, metastasis (TNM) staging system (Figure 1.1.3-2) of the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) is now the standard for colorectal cancer staging. In the TNM system, pathologic staging information derived from surgical resection specimens is considered the most accurate determination of local extent of disease. In rectal cancer, in particular, the macroscopic features of the resection specimen and the microscopic status of the circumferential resection margin are powerful predictors of risk of both local recurrence and overall survival.

The “T” designation refers to the local extent of the untreated primary tumour at the time of diagnosis. The “N” designation refers to the status of regional lymph nodes and “M” refers to distant metastasis. The prefix “p” may be used to imply the pathological determination of the TNM (e.g. pT1) as determined by gross and microscopic examination of the resection specimen of a previously untreated primary tumour. Clinical classification of TNM, giving the “c” prefix (e.g. cT1), is based on

evidence from a variety of techniques such as physical exam, radiological imaging, endoscopy, biopsy and surgical exploration.

Stage	T	N	M
<i>0</i>	<i>Tis</i>	<i>N0</i>	<i>M0</i>
<i>I</i>	<i>T1</i>	<i>N0</i>	<i>M0</i>
	<i>T2</i>	<i>N0</i>	<i>M0</i>
<i>IIA</i>	<i>T3</i>	<i>N0</i>	<i>M0</i>
<i>IIB</i>	<i>T4</i>	<i>N0</i>	<i>M0</i>
<i>IIIA</i>	<i>T1-2</i>	<i>N1</i>	<i>M0</i>
<i>IIIB</i>	<i>T3-4</i>	<i>N1</i>	<i>M0</i>
<i>IIIC</i>	<i>Any T</i>	<i>N2</i>	<i>M0</i>
<i>IV</i>	<i>Any T</i>	<i>Any N</i>	<i>M1</i>

Primary tumour (T):

Tx – primary tumour cannot be assessed

T0 – no evidence of primary tumour

Tis – carcinoma in situ: intraepithelial or invasion of lamina propria

T1 – tumour invasion of submucosa

T2 – tumour invasion of muscularis propria

T3 – tumour invasion through muscularis propria into the subserosa, or into nonperitonealized pericolic or perirectal tissues

T4 – direct tumour invasion of other organs or structures, and/or perforates the visceral peritoneum

Regional Lymph Nodes (N):

Nx – regional lymph nodes cannot be assessed

N0 – no regional lymph node metastases

N1 – metastases in 1-3 regional lymph nodes

N2 – metastases in 4 or more regional lymph nodes

Distant Metastases (M):

Mx – distant metastasis cannot be assessed

M0 – no distant metastasis

M1 – distant metastasis

Figure 1.1.3-2: AJCC TNM staging of colorectal cancer.(46)

A recent modification of the Stage III category, previously a single group, has been made following evidence that the combined depth of involvement and number of involved mesenteric lymph nodes is an important prognostic index. Stage III disease has now been divided into 3 distinct subcategories as follows: Stage IIIA (T1/2, N1), Stage IIIB (T3/4, N1), and Stage IIIC (any T, N2). The 5-year observed survival rate for these three subcategories were 59.8%, 42.0% and 27.3%, respectively.(48) As the Stage III group relies on quantification of mesenteric lymph nodes, it is influenced by the quality of surgical resection and patient factors. The American Joint Committee on Cancer and the College of American Pathologists, and more recently the UK National Institute for Health and Clinical Excellence, recommends that a target of at least 12 lymph nodes need to be examined to determine Stage III identification.(49, 50)

Invasion of the lamina propria (the part of the mucosa surrounding the colorectal crypts) is called *in situ* disease, designated pTis in the TNM classification. Invasion of the submucosa is required to diagnose invasive carcinoma (pT1). The reason for this is that the lamina propria lacks lymphatics, so disease confined to this layer has no means by which to spread.

Lymphovascular invasion is a further important histological prognostic factor, which may occur independently from lymph node invasion. The potential for metastatic spread is increased and T4 tumours with lymphovascular invasion are a high risk group that might benefit from adjuvant chemotherapy.(46)

1.1.4 Modes of spread

Colorectal cancer may spread by local extension, lymphatic spread, haematogenous spread, and by spread through the peritoneal cavity.

Direct extension may occur into any structure intimately related to the position of the colonic tumour. Depending upon the location, this may involve adjacent small intestine, stomach, pelvic organs or abdominal wall, and retroperitoneal extension can invade the ureter, kidney or duodenum.

The lymphatic drainage of the colon follows its blood supply so for caecal tumours, lymph node metastasis occur along the ileo-colic vessels, for tumours of the ascending colon along the right colic, for tumours of the transverse colon along the middle colic and for tumours of the sigmoid and rectum along the inferior colic vessels. Further spread from para-colic nodes eventually reaches the para-aortic nodes in advanced disease. Rectal tumours also drain to lymph nodes within the mesorectum.

Haematogenous spread occurs via the portal system and consequently the liver is at most risk of distant metastasis. The next most common site for haematogenous spread is the lung.

Transperitoneal spread is generally rare for colorectal cancer but if present is associated with an extremely poor prognosis. Mucinous cancers are the most likely to spread in this manner.(51)

1.1.5 Presentation

Colorectal cancer may present with either chronic symptoms or as an emergency. Left sided tumours typically present with a change in bowel habit and rectal

bleeding. Right sided tumours often present late as tumours can grow quite large without causing pain or change in bowel habit due to the liquid consistency of stool and distensible right colon. In these cases, right sided tumours may be detected incidentally as a result of investigations for iron deficiency anaemia. Up to 30% of patients present as an emergency due to obstruction or perforation.(52) Perforation can occur through the tumour itself or proximally at the caecum as a result of a closed loop obstruction when the ileocaecal valve is competent.

1.1.6 Screening

As most cancers arise from pre-existing adenomas, screening has the potential to reduce cancer related deaths. Following the success of a bowel cancer screening pilot study conducted between 2000 and 2007,(53) the NHS Bowel Cancer Screening Programme now offers screening using a guaiac smear faecal occult blood test (Haemoccult) every two years to all men and women aged 60 to 75. A positive test is followed by invitation to attend for colonoscopy. The guaiac test reacts to the peroxidase activity of haem, but this makes the test liable to reaction with other peroxidases in the faeces, such as those from certain fruits, vegetables and red meat. Dietary restrictions are therefore necessary to avoid false positive results. The sensitivity of a onetime test is only 50%–60%. Low sensitivity leads to a high number of false negative results and the impact of receiving a false reassurance. The majority of cases identified by faecal occult blood are false positive cases and these will be subjected to unnecessary further investigations, usually colonoscopy.(54) Nevertheless, the guaiac-based faecal occult blood tests have been shown to reduce colorectal cancer mortality by about 16%,(55) and the main challenge is increasing

screening uptake within the population and increasing the specificity and sensitivity of the screening test. There is increasing interest in faecal immunological testing (FIT) for blood which is reported to have a higher accuracy than the guaiac test. (56) The recent results of a large randomised controlled trial offering once only flexible sigmoidoscopy to people aged between 55 and 64 years showed that after 11.2 years, colorectal cancer incidence was reduced by 23% and mortality by 31% in intention-to-screen analyses and by 33% and 43%, respectively, in per-protocol analyses. Introduction of such a screening programme nationally is now in the pilot phase and may prove to be economically viable given the reduction in incidence.(22)

1.1.7 Diagnosis

Diagnosis should be confirmed by colonoscopy and biopsy of suspicious lesions. A CT colonography may be offered as an alternative mode of investigation for patients with major comorbidity and is largely replacing the practise of flexible sigmoidoscopy and barium enema as it provides a similarly sensitive, less invasive alternative to colonoscopy.(57) The choice is often based on local guidelines and availability. With the exception of patients requiring emergency surgery, computed tomography (CT) of the thorax, abdomen and pelvis, and in the case of rectal cancers magnetic resonance (MR) scans, are used to stage tumours and plan treatment. Patients with early rectal cancers that may be amenable to local excision by MRI criteria are offered endorectal ultrasound scan.(58)

1.1.8 Treatment

1.1.8.1 Surgery

Surgery is the mainstay of treatment for colorectal cancer, and remains the only modality to offer potential cure. Radical resection of the affected segment of colon with its vascular pedicle and accompanying lymphatic drainage is performed. In the case of rectal cancer, resection technique appears to be of great importance. Following colonic resection, most recurrences occur due to disseminated disease, however in rectal cancer, isolated local recurrence tends to occur with wide variation of recurrence rate between individual surgeons.(59) Complete excision of the mesorectum with a clear circumferential resection margin (CRM) is associated with a low rate of local recurrence,(60, 61) illustrating the importance of surgical technique. Despite the removal of visible tumour, cancer may recur in up to half of rectal patients undergoing surgery, usually within 2 years.(62)

The management of stage IV disease is generally palliative with surgical bypass of local obstruction and chemoradiation for local control. In the case of incurable stage IV rectal cancer, removal of the primary tumour may be performed to prevent local invasion of pelvic structures and provide good palliation. With the use of newer generation chemotherapeutic agents, patients with disseminated disease are living longer and may not require palliative surgery for their primary rectal tumour. In a study to examine what happened to the primary rectal tumour in patients with synchronous metastatic disease who were not operated on, the combination of oxaliplatin- or irinotecan-based combination therapy with or without bevacizumab resulted in a median survival of 18 months and 93% of patients never required any

intervention for their primary tumour.(63) This would support a policy of initial non-operative management for asymptomatic rectal cancers with synchronous stage IV disease with the advantage of prompt initiation of systemic chemotherapy. In the case of patients with stage IV colonic tumours and unresectable distant metastases, recent evidence from a multicentre trial suggests a survival advantage for selected patients fit to undergo resection of the primary followed by chemotherapy in association with targeted therapy.(64) However, surgery can be curative in highly selected patients with resectable hepatic or pulmonary metastases.(65, 66) Fit patients with potentially operable metastatic disease of the liver have improved progression free survival after pre and postoperative treatment with FOLFOX4 (Oxaliplatin injection combined with 5-FU and leucovorin) compared with surgery alone.(67)

A summary of current commonly used chemotherapy regimens is displayed in the Appendix to this thesis.

1.1.8.2 Neoadjuvant chemotherapy in colon cancer

Neoadjuvant chemotherapy is not routinely given to patients with colon cancer at the present time. However, the rationale for neoadjuvant chemotherapy in colon cancer appears an attractive one. Adjuvant (post-operative) chemotherapy is not usually started until 2-4 months after the initial diagnosis of cancer and such a delay may reduce the likelihood of chemotherapy eradicating micrometastasis.(68) It is also reported that surgery enhances growth factor activity in the early post-operative period that may promote tumour cell seeding and growth.(69) Thus, pre-operative

chemotherapy can be started earlier and may potentially eradicate micrometastases reducing the risk of local and distant recurrence.

The FOxTROT (“Fluoropyrimidine Oxaliplatin and Targeted Receptor Pre-Operative Therapy”) trial began recruiting patients in 2008 and is designed to evaluate whether giving part of a chemotherapy regimen pre-operatively will improve outcomes and also whether the addition of an EGFR targeted monoclonal antibody (Panitumumab) can increase tumour shrinkage in KRAS wild-type tumours. The study design also includes an assessment of potential biomarkers.

1.1.8.3 Fluorouracil-based adjuvant therapy

Despite potentially curative surgery, around 40% of colorectal cancer patients will eventually relapse. The benefits of a six month adjuvant regimen of bolus 5-Fluorouracil (5-FU) and folinic acid (FA) for stage III disease are well established, with reduction in risk of recurrence and improvements in survival by 5-10 per cent.(70-72) 5-FU, an analogue of uracil, is an antimetabolite drug whose cytotoxicity results from the misincorporation of its active metabolites into RNA and DNA and its inhibition of thymidylate synthase, an important enzyme that catalyses the production of thymidylate, which is necessary for DNA replication and repair.(73) Despite being in use for nearly 50 years in metastatic disease, it was not until the 1990’s that the benefit of 5-FU in the adjuvant setting was firmly established.(74, 75) With only limited response rates as a single agent, 5-FU has remained the main agent for treatment of advanced colorectal cancer due to modulation strategies that have increased its anticancer activity. 5-FU is usually administered with the biomodulator leucovorin (folinic acid), a reduced form of

folate, that enhances the stabilisation of the 5-FU and thymidylate complex, thus enhancing cytotoxicity.(76) Another approach has been the development of Capecitabine, a 5-FU prodrug that is taken orally and is activated within tumour tissue. This avoids the problem associated with intravenous delivery of dihydropyrimidine dehydrogenase (DPD) degradation of 5-FU in the liver resulting in poor bioavailability. Clinical trials with Capecitabine report that it is at least as effective and better tolerated than intravenous 5-FU/FA (Mayo Clinic regimen) in the adjuvant setting,(77) with benefits maintained at 5 years.(78) Capecitabine has not yet been compared with less toxic weekly 5-FU schedules however.

Overwhelming evidence from large multicentre trials, MOSAIC (Multicenter International Study of Oxaliplatin/5FU-LV in the Adjuvant Treatment of Colon Cancer)(79) and National Surgical Adjuvant Breast and Bowel Project (NSABP) C-07,(80) support the use of combination therapy based on oxaliplatin plus 5-FU/FA for stage III disease. Both trials showed similar 20% reduction of the risk of relapse and 15% reduction of the risk of death without increased toxicity. Oxaliplatin is a platinum derivative that forms bulky DNA adducts, and ultimately induces cell death. Importantly, it may also down-regulate thymidylate synthase, thereby potentiating the effect of 5-FU.(81) As a single agent, oxaliplatin has limited efficacy, but is most effective in combination with 5-FU/FA in the FOLFOX regimen.(82) Unfortunately, the addition of Oxaliplatin does come at the price of increased toxicity with significant neutropenia and sensory neuropathy being the biggest problems in the MOSAIC trial. Based on these results, the combination of oxaliplatin and 5-FU/FA has become the standard adjuvant treatment for fit patients with high risk (e.g. heavily node positive) stage III colon cancer who accept the

toxicities. For remaining patients, 6 months of 5-FU/FA or Capecitabine is the preferred option by many oncologists.

The benefit of adjuvant therapy in stage II disease is more difficult to ascertain and markers that could reliably predict response to 5-FU/FA would be particularly useful in this population. A final analysis of mature data from the MOSAIC trial showed no survival benefit in stage II patients,(79) and the UK QUASAR (Quick and Simple and Reliable) trial, despite showing a 22% reduction in recurrence, demonstrated only a modest survival benefit (3.6% improved overall survival) of adjuvant 5-FU/FA,(83) and large pooled analyses have failed to show a significant benefit for 5-FU based chemotherapy for stage II disease,(84) even in those patients with high risk clinicopathological factors.(85) Conventional clinicopathological high risk features for stage II disease include elevated carcinoembryonic antigen (CEA), obstruction or perforation, T4 stage, inadequate nodal resection (<12 nodes), lymphovascular invasion and high grade disease.(84-86) This suggests that the current practise of crude stratification of patients with stage II disease based upon clinicopathological parameters (86, 87) is only able to identify a subgroup of patients who will achieve a small (less than 4%) benefit from treatment. There is a need to identify new prognostic tools that will help identify a high risk subgroup of patients with stage II disease in order to justify the toxicity, inconvenience and cost of adjuvant chemotherapy.(84, 85) At present, patients with stage II disease are individually counselled by an oncologist with regard to their level of risk and the possible benefits of chemotherapy.

In the 2013 national bowel cancer audit, 29,445 patients were diagnosed with colorectal cancer in England and Wales. Two out every five patients did not undergo

surgery for a number of different reasons including early stage of disease, patient frailty, or advanced disease.(88) Around a quarter of those operated will potentially be candidates for adjuvant chemotherapy. Globally, it is estimated that 230,000 cases are eligible for adjuvant chemotherapy.(77) However, the proportion of patients who are deemed suitable for adjuvant therapy but do not receive it is reported from 17-42.5%.(89, 90) Common reasons for failure to receive adjuvant therapy include co-morbid illness, advanced age and patient refusal. In clinical trials of adjuvant therapy, only 58-87%% of patients complete the full course of treatment.(83, 91-93) The potential toxicity of post operative adjuvant chemotherapy must not be underestimated, occurring in over 90% of patients,(92) and these are severe grade 3-4 adverse events in up to 54% of cases.(93) The commonest side effects are gastrointestinal, cardiovascular, dermatological, haematological, and neurologic. Regimens combining 5-FU and folinic acid with oxaliplatin come with the risk of significant neutropenia and sensory neuropathy,(79) the latter of which can be very debilitating and prolonged for many years after completion of treatment.(94) Safety must be a significant consideration in choosing adjuvant chemotherapy, in particular for patients at low risk of recurrence. Further data is required to facilitate the selection of those subgroups of patients whom are likely to receive the most benefit from adjuvant therapy after potentially curative resection of cancer.

1.1.8.4 Adjuvant therapy with biologic targeted agents

Molecular targeted agents have improved the outcome of patients with metastatic colorectal cancer (95) and it was therefore expected that the addition of agents such as bevacizumab might also improve outcomes in stage II and III colon cancers when

used in addition to a standard FOLFOX regimen. Unexpectedly, results from 2 large multicentre trials (National Surgical Adjuvant Breast and Bowel Project (NSABP) C-08 trial and Adjuvant FOLFOX4 Versus Bevacizumab and FOLFOX4 Versus Bevacizumab, Oxaliplatin, and Capecitabine in Patients With High-Risk Stage II or Stage III Colon Cancer (AVANT) trial)(96, 97) suggest that combining bevacizumab with conventional regimens (FOLFOX orXELOX) does not extend disease free survival. Overall, CO-8 found no difference in the proportion of patients who were still alive and free from disease between the control group and the bevacizumab group (75.5% vs 77.4%). Furthermore, the results from AVANT numerically favoured chemotherapy alone (the control arm). Similarly, the North Central Cancer Treatment Group (NCCTG) Intergroup Phase III Trial N0147 (Adjuvant mFOLFOX6 plus or minus cetuximab in patients with KRAS mutant resected stage III colon cancer)(98) showed that addition of Cetuximab led to worse disease free and overall survival with increase toxicity (contrary to its observed benefit in metastatic disease). These data reinforce the notion that primary tumours and metastatic lesions represent distinct diseases that require different treatments.(99) Hypothetically, localised colon cancers may not be as dependent upon signalling via EGFR or VEGF as in the metastatic setting and based upon these reports, Cetuximab or Bevacizumab are not recommended for use with adjuvant chemotherapy.

1.1.8.5 Adjuvant therapy with Irinotecan

Topoisomerase I is an enzyme that catalyses unwinding and rejoining of DNA by single-chain cleavage during DNA replication. The active metabolite of Irinotecan (7-ethyl-10-[4(1-piperidino)-1-piperidino] carbonyloxycamptothecin) SN38 is a

Topoisomerase I inhibitor with antitumour properties.(100) Irinotecan can result in significant improvement in progression free survival and response rate when combined with 5-FU/FA in patients with metastatic colorectal cancer.(101) Yet, as in the case of Bevacizumab and Cetuximab discussed above, this agent performs very differently in the adjuvant setting. Two phase 3 trials, FNCLCC Accord02/FFCD9802(102) and the Cancer and Leukaemia Group B study 89803 (CALGB 89803),(103) failed to show a benefit of adding irinotecan to 5-FU/FA in stage III cancers and in both cases were associated with a significant increase in severe toxicity. As articulated by Saltz et al: “..this demonstrates that advances in the treatment of metastatic disease do not necessarily translate into advances in adjuvant treatment, and it reinforces the need for randomized controlled adjuvant studies.”(103) In another phase 3 trial comparing biweekly infusional fluorouracil (FU) and leucovorin (LV) alone or with irinotecan in the adjuvant treatment of stage III colon cancer, the Pan European Trials in Adjuvant Colon Cancer (PETACC)-3,(93) the primary endpoint of improved disease free survival was not met, and neither was there any benefit to overall survival. Although subgroup analysis revealed a significant advantage for the irinotecan group in relapse-free survival and in disease free survival after adjustment for imbalance of TNM status between groups, the authors were careful not to over interpret these unplanned analyses and concluded that the study did not support the use of irinotecan-based regimens in the adjuvant setting.

1.1.8.6 Palliative chemotherapy

In the palliative setting, before the 1960's patients with metastatic colorectal cancer could not expect to live more than 4-6 months. The introduction of 5-FU and leucovorin improved survival by 3-6 months over best supportive care, as well as improving quality of life.(104) Modern regimens can achieve impressive results. In a randomized multicentre trial, the FOLFOX regimen of oxaliplatin and infused fluorouracil plus leucovorin was shown to deliver a median time to disease progression of 9 months with a median survival of 20 months. FOLFOX was superior to and safer than the alternate regimens that combined irinotecan and oxaliplatin or irinotecan and 5-FU/leucovorin.(105)

The recent development of targeted monoclonal antibodies that may be used in combination with conventional regimens shows promise of further survival benefits. Bevacizumab (Avastin), an antibody to vascular endothelial growth factor (VEGF) confers an additional survival advantage of 4.7 months compared with irinotecan and 5-FU alone and a 4.4 month increase in progression free survival.(95) There have however been reports of increase arterial embolic events associated with Bevacizumab.(106) At present bevacizumab is licensed in the UK in combination with intravenous 5-FU/FA with or without irinotecan for first-line treatment of patients with metastatic carcinoma of the colon or rectum. Cetuximab (Erbix), an epidermal growth factor receptor inhibitor, used alone or in combination with irinotecan in irinotecan-refractory metastatic disease has clinically significant activity,(107) however attempts at combining bevacizumab and cetuximab have resulted in shorter progression-free survival and poorer quality of life.(108) At

present, the use of either bevacizumab or cetuximab is not supported by NICE outside of a clinical trial.(109)

1.1.8.7 Radiotherapy

1.1.8.7.1 Neoadjuvant treatment in rectal cancer

Loco-regional recurrence after surgery for rectal cancer is difficult to treat, and is associated with very distressing symptoms. Preoperative radiotherapy is typically offered to patients with stage II or stage III rectal cancer, however, pelvic radiotherapy is not without its morbidity,(110) so targeting radiotherapy to those at high risk of recurrence is an attractive option. It is also important to acknowledge that any improvement in disease free or overall survival following pre-operative radiotherapy is marginal at best.(111) Accurate pre-operative staging with high-resolution MRI for rectal cancers can help stratify patients with stage II and III disease into those who require neo-adjuvant radiotherapy and those in whom total mesorectal excision alone is adequate to prevent loco-regional recurrence. The Magnetic Resonance Imaging and Rectal Cancer European Equivalence (MERCURY) study group recommend offering neo-adjuvant radiotherapy selectively to patients with MRI-defined poor prognostic factors as follows; potential CRM positive (<1mm), intersphincteric plane involved by tumour (for low rectal cancers), T3 tumour spread more than 5mm, evidence of extramural venous invasion, and lymph node involvement.(112)

Several large trials have shown that local recurrence rates in the region of 5-10% can be achieved with appropriate patient selection. Short course preoperative radiotherapy (SCPRT) followed by TME surgery reduces the risk of local recurrence

(Dutch TME and MRC CR07).(111, 113) SCPRT delivers a lower dose, 25 Gy, but within a short duration of 5 daily fractions over 1 week. Surgery is performed on the following week, before the onset of acute side-effects of radiotherapy. The short interval between commencing radiotherapy and surgery (usually less than 10 days) means that SCPRT does not achieve any significant tumour shrinkage prior to surgical resection and is only appropriate for tumours deemed clinically and radiologically resectable (i.e. CRM not involved). Preoperative long course chemoradiotherapy (CRT) followed by surgery can be used to shrink a tumour prior to resection in the case of a threatened or involved CRM (EORTC 22921; FFCD 9203; GAO/ARO/AIO-94).(114-116) This consists of doses ranging from 45-50 Gy in 25 daily fractions over 5 weeks followed by surgery 4-8 weeks after completion of radiotherapy, allowing maximal tumour shrinkage. This is more effective with the addition of synchronous 5FU-based chemotherapy,(114, 115) which is given either on the first and fifth week of radiotherapy or as a continuous infusion throughout the duration of radiotherapy.

Elderly patients with poor performance status or with other major medical co-morbidities may not be suitable for pre-operative CRT. Some recent data from Uppsala in Sweden(117) and from Leeds(118) suggest that such patients may receive a similar benefit to CRT from short SCPRT followed by an elective delay of 6-8 weeks prior to surgery and this strategy may be a useful alternative in high risk patients with a threatened CRM.

1.1.8.7.2 Postoperative chemoradiotherapy

Involvement of the CRM, defined as tumour present 1mm or less from the radial margin, is associated with a high risk of local recurrence and poor survival.(119) In cases with well established predictive factors of local recurrence (e.g. evidence of tumour at the circumferential resection margin, mesorectal lymph node involvement and extramural vascular invasion), post operative chemoradiotherapy is considered for patients who have not received pre-operative radiotherapy. A dose of 45Gy in 25 fractions over 5 weeks with a planned boost dose of 5.4-9Gy in 3-5 fractions is recommended by The Association of Coloproctology of Great Britain and Ireland.(120)

Post operative chemoradiotherapy has a much smaller effect on local recurrence than preoperative therapy and no effect on disease-free or overall survival. (Colorectal Cancer Collaborative Group, 121)

1.1.9 Biomarkers in colorectal cancer

The era of stratified medicine is upon us and it is important that we choose the right drug for the right patient and reduce treatment failure rates. Colorectal cancer is a complex disease characterised by multiple lesions in key molecular and genetic pathways. Some of these pathways are clinically useful, in particular we are now familiar with treatment pathways such as the EGFR signalling pathway and drugs which target these pathways, such as Cetuximab, are used successfully in advanced disease. However, knowledge of the molecular and genetic makeup of a tumour may also help decide how a particular patient may respond to a certain drug and so called ‘predictive’ pathways are probably more useful than ‘prognostic’ pathways.

At present, the only biomarker sufficiently validated for routine clinical use for determining treatment is KRAS in the setting of metastatic colorectal cancer, with patients who are KRAS wild-type being offered EGFR targeted therapy. But, as will be discussed in section 1.1.9.2, our increasing knowledge of the EGFR-signalling pathways is adding increasing complexity to such decisions, especially in light of recent data regarding the value of other downstream mutations such as BRAF, NRAS and PIK3CA.(122)

An ideal biomarker would be simple, sensitive, specific, inexpensive and reproducible for it to achieve use in routine clinical practise. The Cancer Research UK Biomarker Discovery and Development Committee have defined several categories of biomarkers:

1. Risk assessment/predisposition biomarkers: e.g. APC gene mutations in the diagnosis of FAP.
2. Screening/early detection biomarkers: e.g. FOB testing.
3. Diagnostic biomarkers: e.g. CEA, PSA, CA125 to be used alongside standard imaging.
4. Pharmacological biomarkers: to assess pharmacokinetics or demonstrate clinical effects of the drug, e.g. thiopurine methyltransferase (TMPT) gene and azathioprine treatment in Crohn's disease.
5. Predictive biomarkers: e.g. KRAS and response to EGFR therapy in metastatic colorectal cancer.
6. Prognostic biomarkers: to predict the course of disease and may guide treatment and follow up. No validated prognostic markers exist at present.

This thesis examines the potential of GRP78 as a predictive and prognostic biomarker. The Cancer Research UK Biomarker Discovery and Development Committee have suggested ‘roadmaps’ which define a chronological research pathway for identifying clinically useful predictive biomarkers. The roadmap is illustrated in Figure 1.1.9-1.(123)

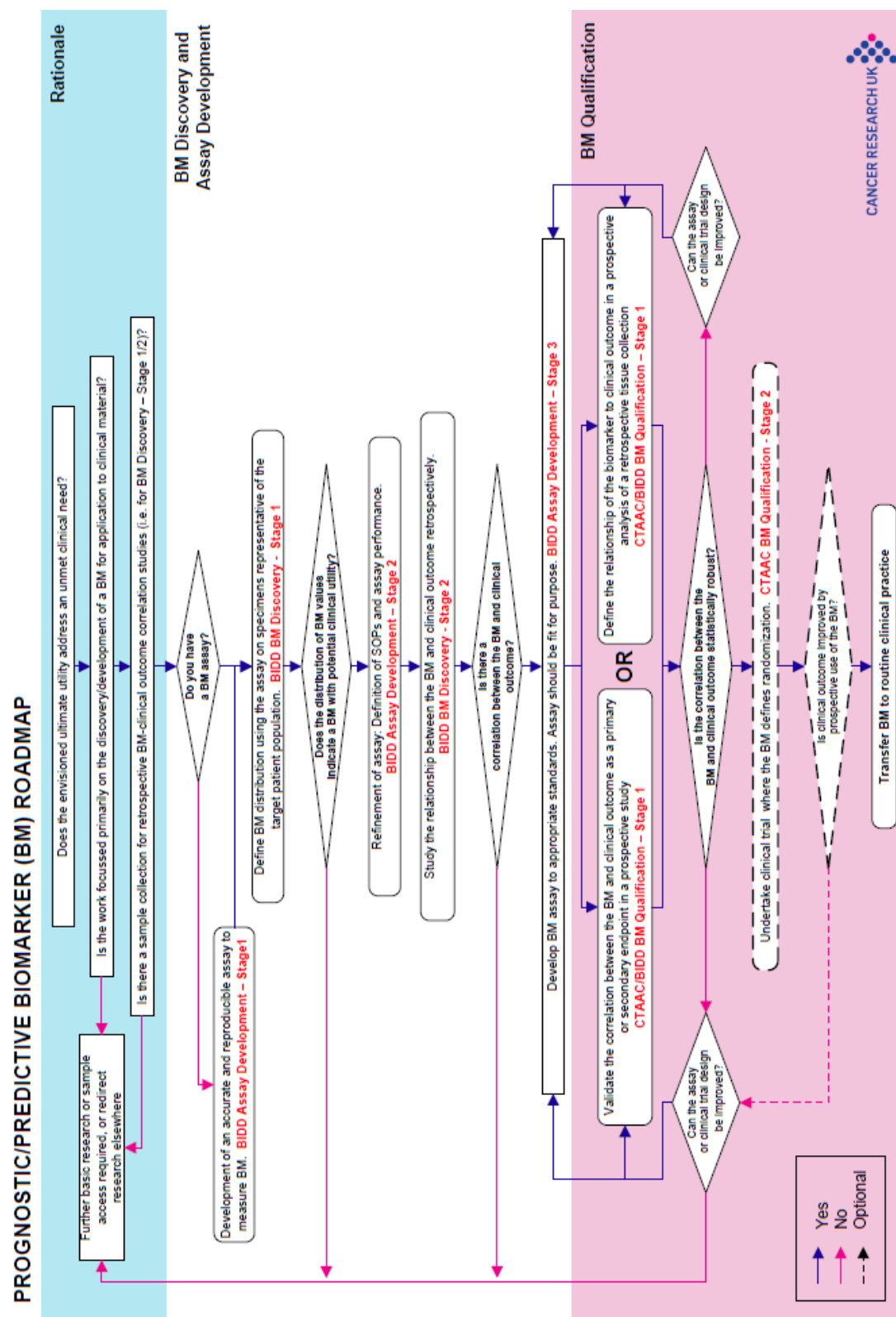


Figure 1.1.9-1: The CR-UK prognostic/predictive biomarker (BM) roadmap.

1.1.9.1 Current status of biomarkers and decision making in the adjuvant setting

The reported lack of benefit from molecular targeted therapy in the adjuvant setting,(124) as discussed in section 1.1.8.4, means that 5-FU-based regimes (FOLFOX) remain the backbone of adjuvant treatment.(87) Below is a summary regarding some of the most promising potential biomarkers to date.

1.1.9.1.1 Thymidylate synthase

Thymidylate synthase (TS) is a target of 5-FU. A number of preclinical biochemical and clinical immunohistochemical and RT-PCR studies have consistently shown that high TS expression predicts a poor response to 5-FU based chemotherapy.(73) However a recent meta-analysis cast doubt on the usefulness of TS as a biomarker for response as the association between high TS and survival was better for those treated by surgery alone than for those who received adjuvant treatment. Furthermore, controversy exists as to the relationship between expression of TS and its relationship to resistance to 5-FU therapy.(31)

1.1.9.1.2 p53 mutation

A potential mode of action of 5-FU may be due to its ability to stabilise p53 function leading to apoptosis.(125) Indeed, a number of studies have shown that disruption of both alleles of TP53 in vitro can make colon cell lines resistant to 5-FU.(126, 127) However, although there is in vitro evidence for p53 involvement in the down-stream response to 5-FU,(73) and p53 mutation has been associated with worse prognosis, reviews of p53 as a potential predictive marker suggest p53 mutation has no effect on outcome in patients treated with 5-FU based chemotherapy.(128)

1.1.9.1.3 18q-LOH/DCC

Loss of heterozygosity on the long arm of chromosome 18 or deleted in colon cancer protein determination by PCR has been implicated as an important step in the development of many colorectal cancers.(24) Some evidence suggests it might be associated with worse prognosis and reduced response rates to chemotherapy.(129, 130) However, it is difficult to draw conclusions from studies investigating chromosome 18q allelic instability as different methodologies and different genetic markers are employed to examine different regions on the chromosome. Additionally, the Pan European Trials in Adjuvant Colon Cancer (PETACC)-3 study identified a stage-specific effect of this biomarker, it showing a prognostic effect in stage III but not stage II disease, as well as revealing that 18q LOH status lost significance when MSI was included in the multivariate analysis suggesting that these markers do not act independently.(131) Therefore, there is insufficient evidence at present to support its routine use.

1.1.9.1.4 DNA mismatch repair and microsatellite instability

As discussed earlier, DNA MMR repairs DNA polymerase mistakes that commonly occur during DNA replication. Affected cells accumulate mutations that drive tumourigenesis and manifest the phenotype of microsatellite instability (MSI), a predisposition to right sided tumours and an unusual histopathological appearance. DNA MMR may also recognise drugs that intercalate with DNA and act as a trigger for apoptosis and thus, alkylation damage as a result of 5-FU incorporation into DNA would not be recognised by a deficient mismatch repair system. MSI may therefore act as a predictor of response to chemotherapy however studies investigating the role

of MSI in response to 5-FU have produced some conflicting data. Despite evidence to demonstrate better prognosis for patients with MSI, there is a body of evidence reporting lack of benefit and worse overall survival following 5-FU.(132, 133) Equally, there are reports that to suggest that benefit from 5-FU is maintained in patients with MSI, although some data suggest that benefit may be limited to those with germline rather than sporadic MSI tumour.(134) Subsequently, at present, the routine use of MSI status to predict response to 5-FU is not supported.(87)

1.1.9.2 Current status of biomarkers and decision making in advanced colorectal cancer

KRAS is an important intermediary in signalling via a number of growth factor receptors, especially epidermal growth factor (EGF) receptor signalling. Epidermal growth factor receptor (EGFR), is an attractive target for cancer treatment because its activation stimulates key processes involved in tumour growth and progression, including proliferation, angiogenesis, invasion, and metastasis.(135) EGFR-targeted monoclonal antibodies, such as Cetuximab and Panitumumab, have been extensively studied in metastatic colorectal cancer and shown to provide modest improvement in overall survival.(136, 137) Positive EGFR expression was initially a criterion for entry into studies evaluating EGFR antibodies, however it soon became apparent that positive EGFR expression was a poor marker for response to treatment as responses were observed in patients with low or negative expression of EGFR.(107, 138-140)

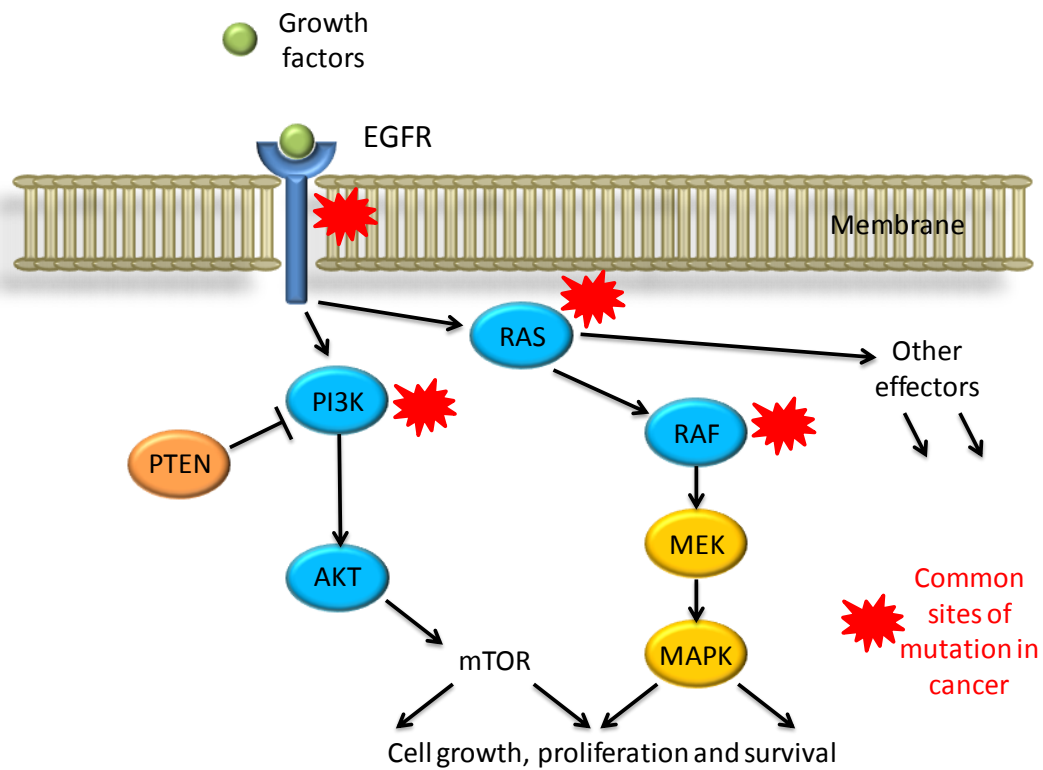


Figure 1.1.9-2: Representation of the EGFR signalling pathway.

Activating KRAS mutations, present in approximately 40% of colorectal cancers,(141-143) result in activation of the EGF signalling pathway via the mitogen activated protein (MAP) kinase pathway at a point downstream of the EGFR (Figure 1.1.9-2). A number of studies have shown that benefit from EGFR antibodies is confined to patients with wild-type KRAS tumours,(143-145) and based on these results, it is now a requirement for patients deemed suitable for EGFR antibodies to undergo KRAS testing and therapy is only approved for KRAS wild-type tumours at present. This is an excellent example of genetic tailoring of treatment in colorectal cancer.

However, not all KRAS mutant tumours are the same. The reality is that response to EGFR targeted therapies based upon KRAS status is actually quite variable and KRAS mutant tumours represent a very heterogenous biological subgroup. Data is

emerging that some KRAS mutant tumours do respond and some KRAS wild-type tumours do not respond to EGFR targeted therapy.

A study of a large cohort of patients treated with cetuximab in the pre-KRAS selection era (2001-2008) reveals a number of other mutations in the EGFR signalling pathway downstream of KRAS that are associated with low response rates. De Roock et al.,(122) report that KRAS mutation was present in 40% of tumours, 14.5% had a PIK3CA mutation, 4.7% had a BRAF mutation and 2.6% had an NRAS mutation. In this cohort, outcome in KRAS wild-type patients was worse in the presence of BRAF, NRAS and PIK3CA exon 20 mutations. The PICCOLO trial (Panitumumab, Irinotecan & Ciclosporin in COLOrectal cancer therapy)(146, 147) was a randomised clinical trial of treatment for fluorouracil-resistant advanced colorectal cancer comparing standard single-agent irinotecan versus irinotecan plus panitumumab and versus irinotecan plus ciclosporin. Following an amendment to protocol in 2008 to include prospective KRAS testing, the trial released results this year which showed a failure to meet the primary endpoint of improved overall survival in KRAS wild-type patients; however a planned biomarker analysis revealed some interesting findings. As expected, progression free survival was improved in patients with KRAS/BRAF wild-type tumours who received panitumumab, with no benefit seen in those patients with KRAS or BRAF mutated tumours. Interestingly, subset analysis revealed that nearly a third of the KRAS wild-type patients were found to have other mutations, thereby conferring drug resistance. Patients with a broadly wild-type profile for KRAS, NRAS, BRAF and PI3K had a good response from panitumumab, but those with a mutation in any of these kinases did not fare as well.

The variable response is further highlighted by additional work by De Roock et al.,(148) revealing better outcomes with cetuximab in patients with p.G13D-mutated tumours than with other KRAS-mutated tumours. In the present context of patients with KRAS codon 12-or KRAS codon 13-mutated tumours being excluded from treatment with cetuximab, this poses some serious questions regarding ongoing randomised controlled trials that are using KRAS status as a discriminator for treatment decisions and suggests that biomarker testing needs to be extended beyond wild-type or mutant KRAS to avoid treatment failure or resistance to EGFR therapy developing.

Whilst many authors have investigated defects in particular molecular pathways, the role of the tumour microenvironment in the behaviour of colorectal cancer is relatively understudied.

1.2 The tumour microenvironment

1.2.1 Aerobic glycolysis

In vivo experimental studies demonstrate important differences in tumour oxygen and glucose consumption. Both human and animal tumours display high levels of glucose uptake and lactate production, even in the presence of adequate oxygen. This phenomenon is termed the Warburg effect or aerobic glycolysis.(149) As a general rule, glycolysis is inhibited in the presence of oxygen in normal cells. Aerobic glycolysis is uniquely observed in cancers. This observation explains the results seen on positron emission tomography (PET) studies with 2-[18F]fluoro-2-deoxy-D-

glucose that consistently demonstrate an elevated glucose uptake in different clinical tumours in vivo. In a series of important experiments, Gullino observed that in tumours transplanted into rats, glucose uptake was far higher than could be predicted by tumour growth or lactate and carbon dioxide (CO₂) elimination. The oxygen consumption of the tumours studied was not deficient when compared with normal tissues with lower oxidative rates and the amount of glucose glycolysed was greater than that oxidised. However, tumours were not able to supplement respiration with glycolysis in the presence of O₂ deficiency.(150) It is increasingly acknowledged that these fundamental principles of tumour metabolism can contribute to malignancy and drug resistance, but importantly, this dysregulated metabolism may also hold the key to therapeutic targeting of tumours.(151, 152)

1.2.2 Response to hypoxia

Early work by Gray identified the importance of tissue oxygenation for sensitivity to radiation damage. Histological assessment of lung adenocarcinoma suggested that due to unrestrained growth, tumour cells are forced away from their supplying vessels resulting in a large diffusion distance for oxygen in respiring tissue.(153) The importance of hypoxia in solid tumours is increasingly recognised in resistance to radiotherapy and chemotherapy.(154, 155)

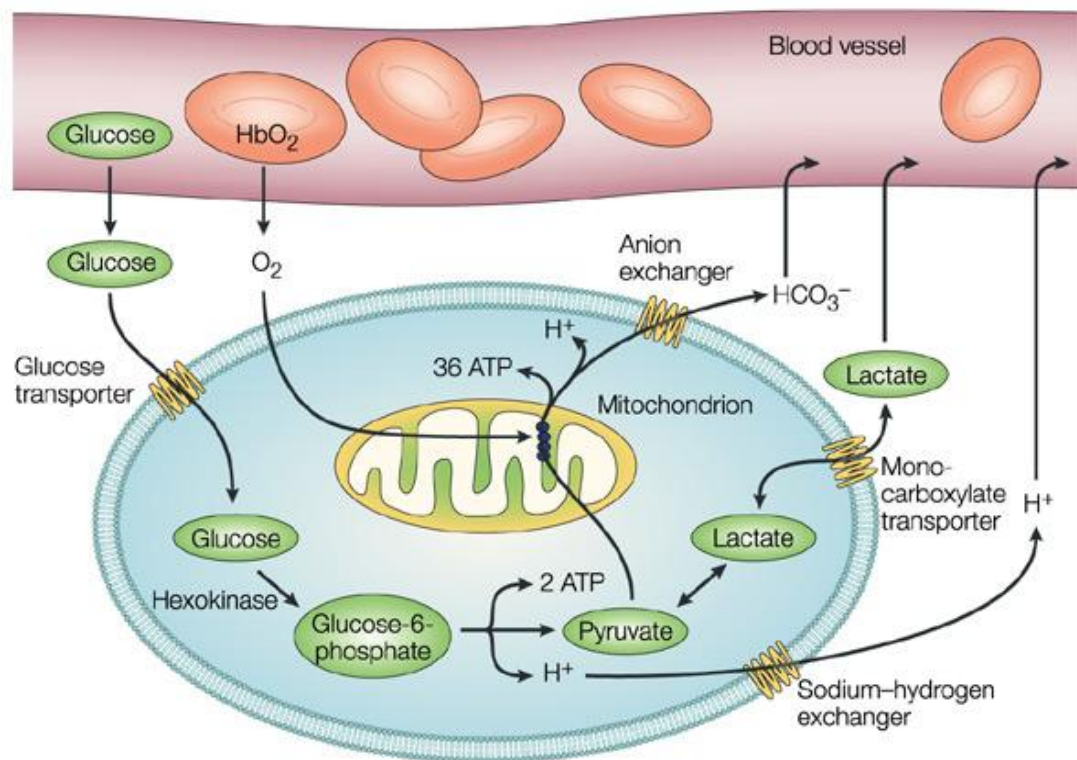


Figure 1.2.2-1: Diagram of glycolysis pathway.(156)

Enlarging tumours may result in an exponential reduction in tissue oxygenation. In experimental mouse tumours, bulky tumours greater than 400mm³ had median pO₂ of less than 10mmHg. At pO₂ below 10mmHg, intracellular acidosis developed and coincided with a reduction in ATP levels, rise in inorganic phosphate and a drop in energy charge.(157) ATP is necessary for normal cell proliferation and survival and comes primarily from either glycolysis, the conversion of glucose to pyruvate in the cytoplasm resulting in a net 2 ATP for each glucose or, from the TCA cycle which uses pyruvate in a series of reactions donating electrons via NADH and FADH₂ to respiratory chain complexes within mitochondria (Figure 1.2.2-1). This process, of which oxygen is the final electron acceptor, generates 36 ATP per glucose molecule. In low oxygen conditions, pyruvate does not enter the TCA cycle and is instead metabolised to lactic acid by lactate dehydrogenase, a process called anaerobic

glycolysis. Cancer cells consume glucose at a high rate and produce lactic acid rather than using the TCA cycle, even in the presence of oxygen.

A normal cellular response to hypoxia is increased glucose utilisation as a result of elevated hypoxia-inducible factor 1 α (HIF-1 α). This in turn induces increased levels of glucose-transporter 1, a transmembrane protein overexpressed in many tumours but undetectable in normal tissues, leading to increased glucose uptake.(158) Under normoxic conditions, HIF-1 α is continuously synthesised and degraded. Hypoxia stimulates anaerobic glycolysis by stabilization of HIF-1 α and its transcription of glycolytic enzyme genes causing increased glucose uptake and lactate production. HIF-1 α binds to the DNA sequence 5'-RCGTG-3' and increases expression of genes encoding glucose transporters and glycolytic enzymes including aldoseA, enolase1, lactate dehydrogenase A, phosphofructokinase L, phosphoglycerate kinase 1 and pyruvate kinase M, as well as angiogenic growth factors (eg VEGF), hexokinase II, and haemopoietic factor.(156) Even in the absence of hypoxia, a variety of oncogenic induced proteins can lead to stabilization of HIF-1 or inhibition of its degradation.(159) Any proliferative advantage conveyed to tumours by aerobic glycolysis is not immediately apparent. In terms of ATP production, anaerobic metabolism of glucose is very inefficient, producing only 2 ATP compared with 38 ATP per glucose with complete oxidation. In fact, this evolutionary adaptation is one of the earliest steps in carcinogenesis and allows tumour cells to survive beyond normal tissue constraints. In the 1960's, Thomlinson and Gray observed that viable tumour cells were not observed greater than 160 μ m from blood vessels. As tumour cells proliferate and expand, the population inevitably moves further away from its blood supply, leading to reduced substrate availability.(156) Tumour angiogenesis is

required to permit further growth in addition to the molecular mechanisms already described. Angiogenic factors, such as VEGF promote increased vascularity of tumours. As new microvessels tend to be highly irregular and tortuous, blood flow and oxygen delivery tends to remain poor and hypoxia is likely to remain a strong selective force.(160) The ability to tolerate transient hypoxia due to the glycolytic adaptation contributes to metastatic potential. The increased presence of $[H^+]$ in the tumour microenvironment is toxic to adjacent normal cells that lack the mechanisms to adapt to extracellular acidosis. This allows tumours to continue to proliferate and may enhance invasive potential and ability to metastasize by causing degradation of the extracellular matrix and promoting angiogenesis.(161)

Anaerobic glycolysis, activated by HIF1 is not sufficient for hypoxic adaptation alone as hypoxic stress results in the production of reactive oxygen species (ROS) by mitochondria that would be toxic were it not for further adaptation. Pyruvate dehydrogenase kinase 1 (PDK1) is a direct HIF1 target gene that inhibits conversion of pyruvate to acetyl-CoA, attenuating mitochondrial respiration and reducing production of ROS. In hypoxic conditions, HIF1 α null mouse embryo fibroblasts fail to activate PDK-1 and undergo apoptosis with a significant rise in ROS. Forced expression of PDK1 by independent retroviral infection increased ATP, prevented hypoxia-induced ROS generation and apoptosis.(162)

The serine/threonine kinase Akt is also associated with increased glucose uptake and aerobic glycolysis independent to HIF1. Akt promotes increased glucose utilization without increasing oxygen consumption. Akt mobilises glucose transporters and activates hexokinase 2 (HK2) to phosphorylate and trap intracellular glucose. Activation of the Akt oncogene is sufficient to cause the switch to aerobic glycolysis

characteristic of cancer cells, but does not increase proliferation of cancer cells in vivo.(163)

In addition to the Atk oncogene, Myc can upregulate the activation of a number of glycolytic enzymes including Glut1,(164) PDK1,(165) LDH-A.(166, 167) Although the Myc transcription factor would appear to enhance the Warburg effect via increasing glycolysis, it has also been shown to encourage respiration in mitochondria leading to increased ROS which would be toxic to tumour cells.(168)

A further adaptation of tumour cells can compensate for this however. One of the most frequently mutated genes, p53, can promote glycolysis by interfering with mitochondrial respiration. Inactivation of p53 in tumour cells can reduce mitochondrial respiration and this effect is mediated by synthesis of Cytochrome C Oxidase 2 (SCO2). SCO2 is necessary for construction of the mitochondrial cytochrome c oxidase (COX) complex, the major site of oxygen consumption in eukaryote cells.(169) Mutant p53 has also been demonstrated to promote glucose influx to cancer cells via increased transcription of type II hexokinase, an enzyme that converts glucose to glucose-6-phosphate, the initial step in glycolysis.(170)

The ability of cancer cells to adapt to the tumour microenvironment by upregulating glycolysis confers an evolutionary survival advantage but overcoming problems with energy production alone is not the only adaptation required by malignant cells.

1.2.3 Cellular stress response

1.2.3.1 Heat shock proteins

Cells exposed to environmental stress are able to demonstrate a coordinated response via inducible gene expression, thus protecting the cell from conditions that would

otherwise lead to irreversible cell damage or death. In the 1960's, an abnormal chromosomal puffing pattern was observed in the salivary glands of heat stressed *Drosophila* larvae.(171) The encoded genes on these chromosomes were later identified as heat shock proteins (hsp's).(172) Members of the heat shock protein family are broadly categorised depending on their molecular weight with the main families being hsp70(70kDa), hsp90(90kDa), hsp100(100kDa), small hsp's and chaperones (groEL/hsp60). They first came to attention due to their induction by cellular thermal stress.(173) Many hsp's also respond under a variety of other stress conditions, including oxygen free radicals, amino acid analogues, ethanol, heavy metals and ischaemia.(174) The function of hsp's is to recognise and stabilise partially folded intermediates during polypeptide folding.(175) In eukaryotic cells exposed to heat shock, heat shock factor (HSF), usually a monomeric non DNA-binding transcription factor, assembles into a trimer and accumulates within the nucleus. Here it binds to, and activates, transcription of heat shock gene promoters and becomes phosphorylated resulting in increased levels of hsp70 and formation of a HSF-hsp70 complex. Prolonged exposure to thermal stress or return to physiological temperatures results in dissociation of HSF from DNA and return to its monomeric form.(176) Hsp's like hsp70 and hsp27 protect against programmed cell death or apoptosis and are found to be overexpressed in a variety of tumours,(174, 177-182) and confer resistance to the adverse tumour microenvironment.(183, 184) Hsp70 can inhibit apoptosis at a number of points on the apoptotic intrinsic pathway including events upstream and downstream of the mitochondria. Upstream, hsp70 can inhibit cytosolic calcium increase,(185) and prevent translocation of Bax to the mitochondria, thus preventing formation of pores in the mitochondrial membrane and

release of cytochrome c.(186) Hsp70 also inhibits cJun N-terminal kinase (JNK) activation,(187) required for Bid-dependent release of cytochrome c from the mitochondria; and stabilises lysosomes, preventing release of lysosomal enzymes into the cytosol that can activate multiple cell death pathways.(188, 189) Downstream of mitochondrial events, hsp70 can modulate function of the apoptosome by interacting with APAF-1.(190) Hsp70 may also inhibit the extrinsic pathway of apoptosis by binding to death receptors.(191)

1.2.3.2 Glucose-regulated proteins

Another family of stress inducible proteins are the endoplasmic reticulum (ER) resident glucose-regulated proteins (GRP's). The GRP's were first reported in the 1970's when it was observed that two proteins of molecular weight 78kDa and 95kDa were produced in increased amounts in chick embryo fibroblasts transformed by Rous sarcoma virus. The increased levels of these proteins was noted to be secondary to depletion of glucose and their synthesis was arrested by restoring glucose to high levels.(192) As the induction of GRP's was primarily observed in conditions that interfere with glycosylation,(193) it was initially thought that GRP's were induced as a result of underglycosylated proteins in the ER.(194) The observation that GRP's were also induced by amino acid analogues,(195-197) did not support this theory. Subsequent experiments revealed that induction of GRP's was triggered by the presence of malformed proteins within the ER and that the induction of GRP78 was not abolished by glucose supplementation. It was further noted that inhibitors of glycosylation within the Golgi apparatus did not induce

synthesis of GRP's, whereas inhibitors of glycosylation in the ER caused significant induction of GRP's.(198)

Apart from glucose deprivation, interference with glycosylation and aminoacid analogues, other stimuli of GRP induction that affect ER function include calcium ionophores,(193, 199, 200) Thapsigargin (an ER calcium ATPase inhibitor),(200) tunicamycin,(198) sulfhydryl-reducing agents,(159) low extracellular pH and hypoxia.(201)

1.3 GRP78 and the Unfolded Protein Response

1.3.1 GRP78

The best characterised GRP is GRP78, also known as BiP, the immunoglobulin heavy chain-binding protein. GRP78 resides primarily in the ER, an essential perinuclear organelle for the synthesis and folding of secretory and membrane proteins, which accounts for about one third of the cell's proteins. In addition to facilitating proper protein folding, preventing intermediates from aggregating, and targeting misfolded protein for proteasome degradation, GRP78 also binds Ca^{2+} and serves as an ER stress signalling regulator.(202) BiP was identified in 1983 when it was observed that immunoglobulin heavy chain synthesised by pre-B cell lymphocytes bound to a protein that inhibited their secretion in the absence of light chains, thus preventing secretion of incompletely folded immunoglobulins.(203) GRP78 and BiP were later found to be identical ER-resident proteins that were abundant in antibody-secreting cells.(204) GRP78 is very similar to hsp70, sharing about 60% of its amino acid sequence including the ATP-binding site required for

their role as a chaperone in protein folding. GRP78 differs from hsp70 in two important aspects. First, GRP78 is primarily an ER resident protein whereas hsp70 is cytosolic and, second, thermal stress does not significantly induce GRP78.(205)

1.3.1.1 Atypical GRP78

1.3.1.1.1 Cell surface GRP78

Although GRP78 is generally regarded as an ER resident chaperone protein, there is mounting evidence that GRP78 is also located on the cell surface of cancer cells but not normal cells, and that it can act as a receptor that regulates cellular activity.(206) In 1998, cell surface localized GRP78, induced by thapsigargin treatment in human rhabdomyosarcoma cells, was confirmed by biotinylation and chromatography.(207) In an additional study, GRP78 was found to be expressed on the cell surface with MHC class1 in unstressed cells. GRP78 is known to associate with MHC class 1 heavy chain intracellularly during folding and assembly, but on the cell surface GRP78 does not appear reliant on MHC class 1 and, in fact, in the absence of MHC class 1, GRP78 is overexpressed.(208)

In prostate cancer cells, GRP78 acts as a high-affinity receptor for the protease inhibitor alpha-2-macroglobulin ($\alpha 2M^*$) and binding of $\alpha 2M^*$ to cell surface GRP78 can promote cellular proliferation.(209-212) In a variety of cancer cell lines, global profiling of the cell surface proteome revealed that GRP78, among other chaperone proteins, was present in all cell types tested in relatively high abundance.(213) Circulating antibodies to cell surface GRP78 in the serum of prostate cancer patients correlates with more aggressive cancer phenotype and reduced survival. As proof of principle that cell surface GRP78 may have potential therapeutic value, a synthetic

chimeric peptide with GRP78 binding motifs fused to proapoptotic sequences could specifically target tumour cells in vivo with minimal effect on normal tissues and in human cancer cells ex vivo, confirming that cell surface GRP78 is a functional molecule leading to internalization of GRP78 binding ligands.(214, 215)

The question of how an ER resident protein such as GRP78 can localize to the cell surface is an interesting one. It has been shown that the murine tumour cell DnaJ-like protein-1 (MTJ-1), as well as being a transmembrane protein, also acts as a co-chaperone to GRP78 within the ER.(216) MTJ-1 co-immunoprecipitates with GRP78 in the plasma membranes of macrophages and silencing the MTJ-1 gene expression by RNA interference abolishes cell surface localization of GRP78; thus suggesting that MTJ-1 is essential for transport to the cell surface.(217) Another theory is that GRP78 is expressed to such high levels in tumours that it may oversaturate the ER protein retrieval mechanism, resulting in cotrafficking to the cell surface with its client proteins.(218, 219) In principle, drugs targeting cell surface GRP78 may have antitumour properties with minimal adverse effects on normal organs and tissues.

1.3.1.1.2 Cytoplasmic GRP78

GRP78va is a novel cytosolic isoform devoid of an ER signal sequence. It is generated through ER stress-induced alternative pre-mRNA splicing, a process through which a single gene may generate several distinct protein isoforms that may have diverse or even antagonistic functions.(220, 221) It is known to be elevated in leukaemic cell lines and in leukaemia patient samples and specifically enhances PERK signalling to promote cell survival under conditions of ER stress. It is not known which client proteins it interacts with in the cytosol.

1.3.1.1.3 Mitochondrial GRP78

Cellular fractionation and protease digestion of isolated mitochondria suggested that a significant amount of GRP78 was localised to the mitochondria in ER stressed cells. This was confirmed by immunoelectron microscopy, demonstrating that during UPR activation, GRP78 localised to the mitochondrial membrane compartment.(222) Although its functions at this site are not yet understood, it is possible that it may modulate the mitochondrial component of the intrinsic apoptosis pathway.

1.3.2 'ER' Stress

The induction of GRP78 is an important response to disturbance of ER homeostasis.(223) The ER is a unique oxidising compartment for the folding of membrane and secretory proteins, and has a role as a major signal-transducing organelle. It also provides a highly selective quality control system to ensure correct protein folding and assembly, and recognise unfolded proteins so that they can be repaired or targeted for proteasome degradation. A number of biochemical, physical and pathological stimuli, for example, ER calcium depletion, glucose deprivation, oxidative stress and DNA damage can disrupt ER homeostasis and lead to accumulation of unfolded or misfolded proteins in the ER – so called 'ER stress'. Eukaryotic cells have evolved a coordinated cellular response to such stress termed the unfolded protein response (UPR).(198, 219, 224, 225)

1.3.3 The Unfolded Protein Response

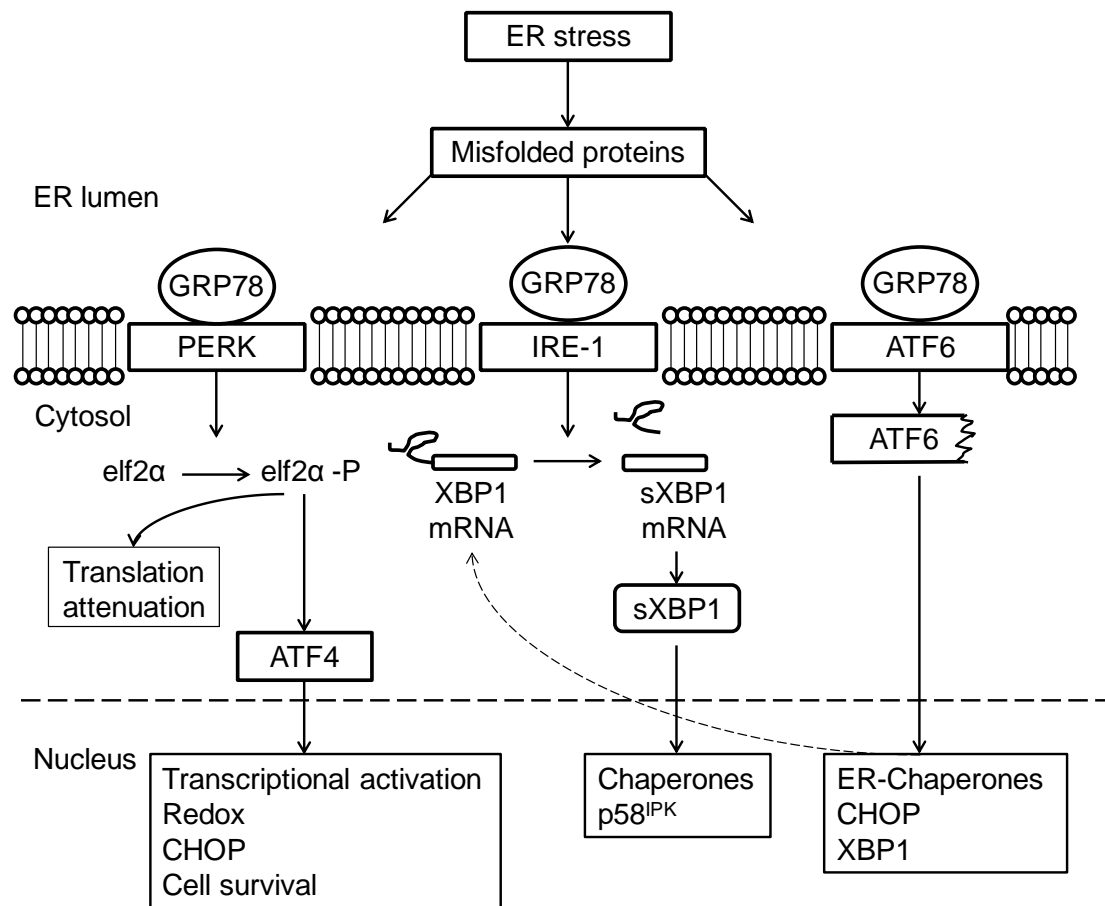


Figure 1.3.3-1: Endoplasmic reticulum (ER) stress-mediated unfolded protein response (UPR) signal pathways.

In unstressed cells, GRP78 interacts with the luminal domain of IRE-1, PERK and ATF6, and negatively regulates them. Increased binding of GRP78 to unfolded proteins releases binding of IRE-1, PERK and ATF6 leading to activation of the UPR. Dissociated ATF6 is released from the ER and moves to the Golgi apparatus where it is cleaved by the proteases S1P and S2P, yielding an active fragment ATF6f that translocates to the nucleus, and binds to and activates the ER stress response element (ERSE) found on the promoters of various UPR target genes including GRP78, CHOP and XBP1. PERK regulated phosphorylation of eIF2α attenuates mRNA translation apart from one mRNA encoding ATF4 which requires eIF2α phosphorylation. ATF4 activates genes encoding proteins involved in aminoacid biosynthesis, antioxidative stress response, and ER stress-induced apoptosis. IRE1 homodimerizes and becomes autophosphorylated leading to activation of its endoribonuclease function. The endoribonuclease activity is responsible for promoting increased splicing of X-box binding protein 1 (XBP1). This longer XBP1 protein is a potent transcriptional activator of UPR target genes involved in protein

folding and degradation of misfolded proteins. There is a delay of the IRE-1 pathway relative to that of PERK and ATF6 as the substrate XBP1 mRNA is expressed at low levels in non stressed cells, but is upregulated by ATF6 in the presence of ER stress. These actions aim to restore ER function by blocking further accumulation of protein, enhancing folding capacity and initiating degradation of protein aggregates. Modified from (226)

1.3.3.1 Cell survival/adaptation

Tumorigenesis and proliferation of cancer cells requires increased protein synthesis, a physiological ER stress, which together with nutrient deprivation and hypoxia of the growing tumour causes further accumulation of unfolded proteins in the ER. The importance of the UPR in carcinogenesis, metastasis and drug resistance has become well recognised and intensely studied.

A key initiator of the UPR is GRP78. The ability of GRP78 to dissociate from several important ER-resident transmembrane proteins under conditions of ER stress leads to a cascade of signal transduction pathways that modulate cell survival or, if the stress is significantly severe, apoptosis.(227-229) These pathways prevent accumulation of unfolded proteins in the ER by reducing the protein-folding load, increasing the ER protein-folding capacity, and increasing degradation of misfolded proteins through ER-associated protein degradation (ERAD) or autophagy.(230-232) This network of responses to ER stress is regulated by only three ER transmembrane proteins: inositol-requiring 1 alpha (IRE-1), double strand RNA-activated protein kinase-like ER kinase (PERK), and activating transcription factor 6 (ATF6).(231) Remarkably these 3 protein sensors are activated by a single common stimulus, the accumulation of misfolded proteins in the ER. Each sensor has an ER luminal domain sensitive to accumulation of misfolded proteins, an ER transmembrane domain that can target protein for localization to the ER membrane, and a cytosolic

functional domain.(233) In unstressed cells, GRP78 interacts with the luminal domain of IRE-1, PERK and ATF6, and negatively regulates them. The luminal domains of IRE-1 and PERK receptors that bind GRP78 are interchangeable,(234-237) and importantly, the peptide-binding site of GRP78 that associates with the receptors is the same that binds to hydrophobic patches of misfolded or unfolded ER peptides.(229) Activation of the UPR occurs by dissociation of GRP78 from IRE-1, PERK and ATF6, possibly as a result of GRP78 preferentially associating with misfolded nascent protein rather than these 3 receptors. Increased binding of GRP78 to unfolded proteins releases binding of IRE-1, PERK and ATF6 leading to activation of the UPR. IRE-1 and PERK, once released from GRP78, homodimerise through their luminal domains, autophosphorylate their cytosolic domains and become active.(234) Dissociated ATF6 is released from the ER and moves to the Golgi apparatus where it is cleaved by the proteases S1P and S2P, yielding an active fragment ATF6f that translocates to the nucleus, and binds to and activates the ER stress response element (ERSE) found on the promoters of various UPR target genes including GRP78, CHOP and XBP1.(238-241)

Following ER stress, the first pathway to be activated, within minutes,(242) is the PERK regulated phosphorylation of the α subunit of eukaryotic translation initiation factor (eIF2 α) on serine 51. This interferes with global mRNA binding of ribosomal 60s and 40s subunits, via interference with the formation of a 43s initiation complex, causing translational attenuation and reduction of protein synthesis, and thus reduced ER workload.(243-246) Although in general, phosphorylation of eIF2 α attenuates mRNA translation, one mRNA encoding ATF4 requires eIF2 α phosphorylation.(247) ATF4 activates genes encoding proteins involved in aminoacid biosynthesis,

antioxidative stress response, and ER stress-induced apoptosis.(248) In view of the action of the PERK/eIF2 α pathway, strict control of the phosphorylation of eIF2 α is necessary for the cell to survive. In fact, the activation of PERK by ER stress is rapidly reversible with activated PERK being rapidly dephosphorylated upon restoration of ER homeostasis. Phosphorylation of eIF2 α is also controlled by a negative feedback mechanism. Two inhibitory genes are growth arrest and DNA-damage-inducible protein 34 (GADD34) and constitutive repressor of eIF2 α phosphorylation (CReP). Both GADD34 and CReP activate two phosphatase complexes that dephosphorylate eIF2 α . CReP is constitutively expressed at baseline and GADD34 is induced by eIF2 α phosphorylation and serves in a negative feedback loop.(234, 249, 250)

After PERK activation and reduction of protein synthesis, ATF6 cleavage occurs next most rapidly. The relative delay is caused by the need for nuclear translocation of ATF6f and the induction of transcription and protein synthesis. Activation of ATF6 is thought to occur prior to XBP1 activation,(251) through ATF6 translocation to the Golgi apparatus from the ER lumen where Golgi-resident proteases cleave the protein to release a free-cytosolic DNA-binding portion. This form of ATF6 translocates to the nucleus and acts as a transcription factor for genes that augment protein folding capacity, including GRP78.(238, 239, 241)

IRE-1 is a type 1 transmembrane Ser/Thr protein kinase that can also function as an endoribonuclease (RNase).(252, 253) In mammals, two homologous genes encode for IRE-1; IRE-1 α is expressed in all cells, and IRE-1 β which is found primarily in intestinal epithelial cells.(253) In response to ER-stress and subsequent release from GRP78, IRE1 homodimerizes and becomes autophosphorylated leading amongst

other events to activation of its endoribonuclease function. The endoribonuclease activity is responsible for promoting increased splicing of a non-conventional 26-base intron from the XBP1u mRNA that encodes a truncated 271 amino-acid isoform of the X-box binding protein 1 (XBP1).(254) Splicing by IRE1 results in expression of a longer XBP1 protein isoform of 376 amino acids as a result of a frameshift in the fully-spliced mRNA (XBP1s) and this longer XBP1 protein is a potent transcriptional activator of UPR target genes involved in protein folding and degradation of misfolded proteins.(255) There is a delay of the IRE-1 pathway relative to that of PERK and ATF6 as the substrate XBP1 mRNA is expressed at low levels in non stressed cells, but is upregulated by ATF6 in the presence of ER stress.(254) Therefore, XBP1 mRNA is not produced in large amounts until after the initial induction of PERK and ATF6 UPR pathways. Spliced XBP1 mRNA encodes a potent basic leucine zipper (bZIP)-containing trans-activator that induces expression of a group of ER chaperones and enzymes that assist with protein folding, secretion, and degradation, including genes encoding XBP1, GRP78 and the hsp40 like p58^{IPK} which can interact with PERK and down regulate its phosphorylation. It has been proposed that as p58^{IPK} is produced relatively late in the UPR pathway, it may serve as a limiter to turn off the UPR when the ER stress has been overcome.(229, 255)

1.3.3.2 ERAD

Another key component of the UPR is the targeting of unfolded proteins for degradation via the ER-associated degradation (ERAD) pathway.(256, 257) Unfolded proteins are selected by molecular chaperones and targeted for

retrotranslocation to the cytoplasm. The central component of the translocon, Sec61p, forms a transmembrane channel that, in the presence of GRP78, allows retrograde transport of proteins targeted for ERAD from the ER to the cytosol. After transport, substrate molecules are ubiquitinated prior to proteolysis by the 26S proteasome.(258) Virtually all ERAD substrates are modified with ubiquitin, via ER ubiquitin ligases located in or near the ER membrane.(259)

Although ERAD is a normal part of ER quality control, genes involved in ERAD are upregulated as part of the UPR by the IRE1/XBP1 pathway. The IRE1 pathway mediates induction of EDEM (ER degradation-enhancing α mannosidase-like protein).(251) EDEM is involved in identification and targeting of unfolded protein for degradation.(260)

1.3.3.3 Apoptosis

In addition to supporting adaptation to, and recovery from ER stress, the UPR will also initiate apoptotic pathways if the cell is unable to adapt or the stress is prolonged. Apoptosis is an essential mechanism if disease resolution is to occur.

The initiation of apoptosis is classically divided into intrinsic and extrinsic pathways. The intrinsic pathway responds to intracellular insults, such as DNA damage, and results in a balance shift between proapoptotic BH3-only proteins, e.g., Bad, Bak or Bax, and antiapoptotic proteins, e.g., Bcl-2 proteins. Bak and Bax act on the mitochondrial membrane to form pores, resulting in release of cytochrome-c into the cytosol. Cytochrome-c mediates the formation of a complex between Apaf-1 and procaspase-9, leading to activation of caspase-9 and subsequently the executioner caspase-3.(229) The extrinsic pathway responds to extracellular stimulus, e.g., ligation of tumour necrosis factor- α , leading to self-association of cell surface receptors, recruitment of caspases, and activation of a caspase-cascade resulting in apoptosis. The division between intrinsic and extrinsic pathways in ER-stress is not clear cut however, with much overlap. In fact, ER-stress is better described as an intrinsic apoptotic signal rather than extrinsic.(229)

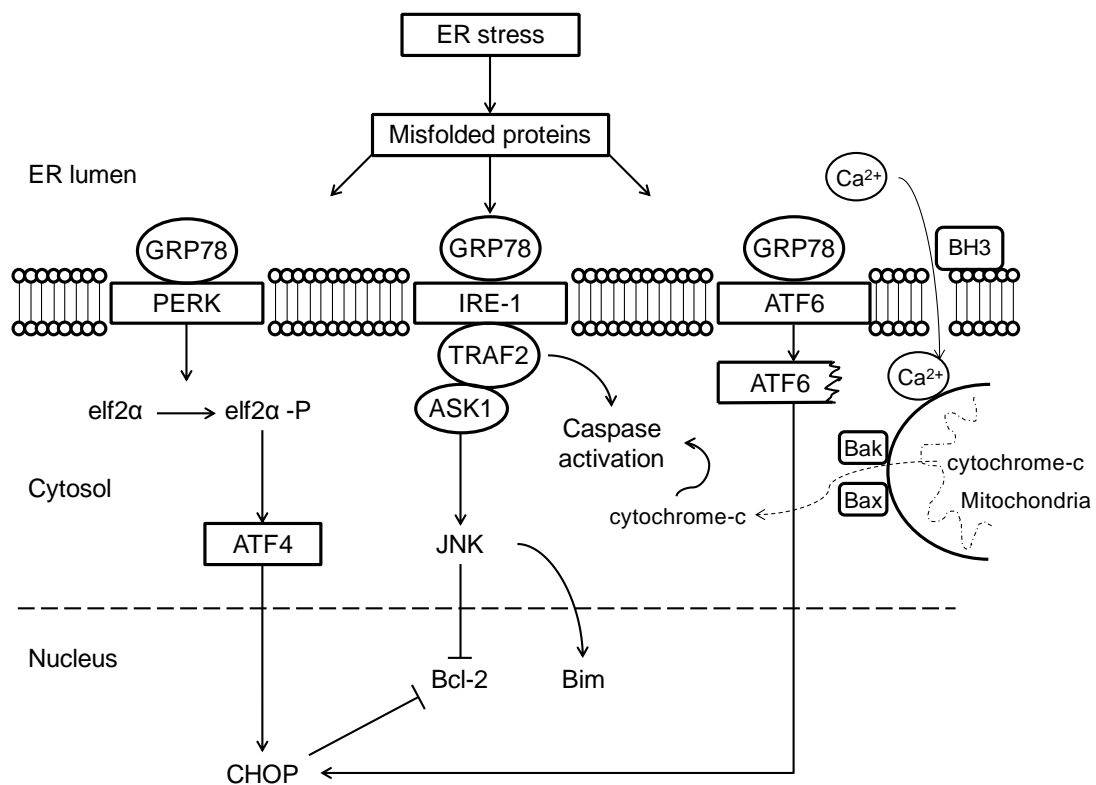


Figure 1.3.3-2: Simplified representation of mammalian ER stress-induced apoptotic pathways.

ER stress activates mitochondria-dependent and mitochondria-independent apoptotic pathways. ATF4 promotes transcription of CHOP, a negative regulator of the anti-apoptotic Bcl-2. In addition, activation of IRE-1 recruits TRAF2 and activates ASK1 forming a trimeric complex, which activates pro-apoptotic JNK-mediated signalling pathways. JNK promotes cell death by phosphorylation of Bcl-2 and activation of pro-apoptotic Bim. TRAF2 may also promote caspase activation independent of mitochondrial cytochrome-c release. ER stress-induced conformational change in Bcl-2 family proteins including Bax and Bak leads to calcium depletion from the ER. This calcium flux activates mitochondrial cytochrome-c release and caspase activation.

1.3.3.3.1 PERK/eIF2α mediated apoptosis

Under conditions of prolonged ER stress, PERK phosphorylates eIF2α leading to attenuation of mRNA translation in general, but a few specific mRNAs require eIF2α phosphorylation for efficient translation. The best described of these is ATF4, a cAMP response element-binding transcription factor (C/EBP). ATF4 induces

transcription of C/EBP homologous protein (CHOP), also known as GADD153, a known pro-apoptotic factor (261) (note that ATF6 can also induce the CHOP gene).(262) CHOP reduces transcription of anti-apoptotic Bcl-2,(263) promotes depletion of cellular glutathione, and increases production of reactive-oxygen species causing release of cytochrome-c from mitochondria causing apoptosis. CHOP also activates GADD34 which dephosphorylates eIF2 α , thus removing the brake from protein synthesis leading to further overloading of the already stressed ER. CHOP further promotes oxidising conditions by activating ERO1 α , an ER oxidase that causes hyperoxidization of the ER.(264)

1.3.3.3.2 IRE-1 α mediated apoptosis

In addition to its endoribonuclease activity, IRE-1 α can serve as a scaffold protein that recruits tumour necrosis factor (TNF)-receptor associated factor 2 (TRAF2).(265) This interaction recruits c-Jun NH2-terminal inhibiting kinase (JIK) which, through interaction with IRE-1 α and TRAF2, activates JUN N-terminal kinase (JNK)-mediated signalling pathways.(265, 266) IRE-1-TRAF2 reaction also activates apoptosis signal-regulating kinase-1 (ASK1). Formation of a trimeric complex between IRE-1 α , TRAF2 and ASK1 activates ASK1 and JNK.(267) JNK can promote cell death by phosphorylation of Bcl-2 and BIM causing apoptosis.(268) TRAF2 promotes clustering of murine procaspase-12 and during ER stress, cleavage occurs releasing procaspase-12 for activation, presumably as a result of TRAF2 recruitment by IRE-1 α .(266) In humans, the caspase-12 gene is frequently inactive and it is proposed that caspase-4 mediates ER stress induced apoptosis in humans.(269) This pathway is independent of mitochondrial cytochrome-c release.

The disruption of intracellular calcium homeostasis is an additional trigger of apoptosis during ER stress. The Bcl-2 family proteins, including Bax and Bak, are associated with mitochondrial and ER membranes and undergo conformational change and oligomerization during ER stress. This leads to calcium depletion from the ER, inducing caspase-12 cleavage.(270) Cytosolic calcium activates m-calpain, a calcium dependent cysteine protease, which cleaves and activates procaspase-12. In addition, mitochondrial Bak enhances caspase-7 and PARP cleavage, leading to release of cytochrome-c. Caspase-7 can translocate from the cytosol to the ER surface and activate caspase-12, in addition to the activation by calpains.(271)

Despite an increased understanding of the apoptotic pathways involved in ER stress, the control between adaptation and recovery, and apoptosis remains unclear.

1.3.4 GRP78 and malignancy

The UPR is essential for the survival of cancer cells through adaptation to hostile conditions, and is associated with upregulation of GRP78. Evidence that GRP78 is required for tumour growth came from the observation that GRP78 knockdown fibrosarcoma cells were unable to form tumours or they quickly regressed in vivo.(272) Increasing evidence suggests that GRP78 can play an important cytoprotective role in cancer as well as conferring drug resistance.(202, 218, 273) In addition to activation by the UPR, c-Myb, a transcription factor frequently overexpressed in colon cancer cell lines, can bind to sequences within and modulate the human GRP78 promoter, independent of UPR sequences like the ERSE motif.(274)

GRP78 has been found to be highly expressed in a number of different cell lines and solid tumours, and correlates with malignancy, metastasis and drug resistance.(223) In head and neck cancer (HNC) cell lines, GRP78 is highly expressed. Knockdown of GRP78 by small interfering RNA (siRNA) significantly reduced cell growth and colony formation, and inhibited migration and invasive ability. Subsequent xenograft study demonstrated that administration of a siRNA plasmid into HNC xenografts inhibited tumour growth in situ and liver metastasis.(275) Overexpression of GRP78 in gastric cancer correlates with tumour size, depth of invasion, lymphatic and venous invasion, lymph node metastasis and stage of disease,(276) is inversely correlated with patient survival and GRP78 knockdown in gastric cancer xenograft mouse model inhibited tumour growth and metastasis.(277) In hepatocellular carcinoma, high GRP78 expression correlates with increasing histological grade (278). GRP78 expression in prostate tumours is markedly higher than in benign prostate tissue and the degree of expression correlates with increased risk of recurrence, reduced survival, and is also associated with the development of castration resistance.(279, 280) In patients with diffuse large B-cell lymphoma, overexpression of GRP78 was associated with worse overall survival and characterising tumours by GRP78 may thus be of value in selecting patients for combination therapy.(281)

The observation that GRP78 is elevated in malignant tumours and metastasis suggests a role for GRP78 in improving cell motility, as well as protecting it from the hostile host environment. As discussed earlier, $\alpha 2M^*$ can bind cell surface GRP78 in 1-LN human prostate cancer cells.(211) Alpha 2M* can activate 21-kDA activated kinases (PAK's), which are important for cell motility(282), oncogenesis and

metastasis.(283) Metastatic potential may also be enhanced by the PERK/eIF2 α mediated G1 cell cycle arrest, via cyclin D1 attenuation. This allows tumours to remain dormant until such a time that local conditions may improve.(284) This mechanism is also likely to contribute to drug resistance as many anti-cancer drugs act on dividing cells and will therefore be ineffective against dormant cells.

While the majority of evidence suggests that GRP78 may predict for a poor prognosis in solid tumours, a few exceptions do exist. There is no evidence of a correlation between GRP78 and pathological stage in lung cancer and, in fact, patients with positive GRP78 expression tend toward better prognosis.(285) In neuroblastoma, GRP78 expression is higher in more differentiated tumours and correlates with better survival for patients with both well and poorly differentiated tumours.(286) In primary resected oesophageal adenocarcinoma, GRP78mRNA is more elevated in early pT1 cancer compared with more advanced stages or normal oesophageal squamous epithelium. Survival analysis suggests a trend to better survival from oesophageal cancer in those patients with tumours expressing higher levels of GRP78.(287)

1.3.4.1 GRP78 and chemosensitivity

Overexpression and siRNA-mediated knockdown studies have established that GRP78 can convey resistance to a variety of anticancer therapy in tumours.(218) The antiapoptotic effects of GRP78 affect the efficacy of anticancer drugs, many of which act through inducing ER stress.

Topoisomerase II α (topoII α) is an ATP-dependent nuclear enzyme important for DNA replication and is therefore an attractive target for actively dividing cancer

cells. TopoII α active drugs such as etoposide (VP-16) become less effective with increasing GRP78 expression resulting in reduced topoII α activity, although VP16 itself does not increase GRP78 as it does not cause ER stress. Thus, in solid tumours with hypoxic cores that result in upregulation of GRP78, development of resistance to TopoII α inhibitors is likely.(288) The mechanism of etoposide resistance is now understood. Etoposide induced cell death occurs via the caspase signalling cascade, in particular the activation of caspase-7, which is localized to mitochondrial and ER membranes. GRP78 overexpression suppresses caspase-7 activity via the ATP binding domain. Of further interest is the finding that unlike standard UPR inducers such as tunicamycin, that alter drug sensitivity by causing cell cycle arrest in G1, GRP78 overexpression does not cause any alteration of the cell cycle.(289) Caspase-7 is the downstream executioner caspase for a number of anticancer drugs and GRP78 possibly confers general resistance to cancer cells.(290)

Cisplatin is a platinum based chemotherapy drug that binds to and causes cross-linking of DNA. In contrast to etoposide resistance, activation of the UPR, by pre-treatment with 6-AN in colon cancer cell lines, cause increased sensitivity to DNA cross linking agents such as Cisplatin, melphalen and 1,3-bis(2-chloroethyl)-1-nitrosurea (BCNU). It was shown that sensitisation was independent of the nature in which GRP78 was up-regulated and that the mechanism for sensitization was impairment of DNA cross-link repair. It is proposed that when overexpressed, some GRP78 can translocate to the nucleus where it attenuates the repair of DNA cross-links.(291, 292)

By contrast, other investigators propose that GRP78 might contribute to resistance to Cisplatin. In enucleated cells, Cisplatin induced calpain-dependent activation of ER

caspase 12 and increased expression of GRP78, both markers of ER stress. Cisplatin can therefore be considered as an ER-active anticancer drug. Increase in GRP78 may convey an antiapoptotic or protective effect contributing to cisplatin resistance.(293)

Drugs such as combretastatin A4P (CA4P), which targets proliferating immature endothelial cells, have selective effects of destroying tumour vasculature, in particular the central, poorly perfused hypoxic regions. However, this can have the unintended effect of triggering a prosurvival response such as GRP78 expression. In a xenograft human breast cancer model, treatment with CA4P, and contortostatin, another antiangiogenic drug with anti-tumour properties, promoted transcriptional activation of GRP78 and elevated GRP78 protein in the surviving tumour cells, resulting in drug resistance by upregulating survival pathways.(294)

Histone deacetylase (HDAC) inhibitors are promising drugs that cause increased differentiation, growth arrest and apoptosis. HDACi's induce GRP78 but not other stress response pathways. Overexpression of GRP78 confers resistance to HDACi-induced apoptosis in cancer cells, and knockdown of GRP78 by siRNA sensitizes cells to HDACi's.(295)

In human breast cancer, oestrogenic stimulation increased GRP78 in vitro and activated the downstream UPR regulator XBP-1. Furthermore, UPR induction by glucose deprivation enhanced resistance to 5-fluorouracil and doxorubicin.(296) It is also suggested that GRP78 can contribute to resistance of breast cancers to anti-oestrogen therapy by suppressing intrinsic apoptotic pathways.(297)

1.3.5 Therapeutic implications

In view of the weight of evidence in support of UPR involvement in solid-tumour development and drug-resistance, targeting of the UPR and in particular GRP78 becomes an attractive approach to develop new anticancer therapies.

The observations that silencing GRP78 by siRNA or antisense can reverse malignant phenotypes suggest GRP78 as a potential anticancer target.(272, 294, 295, 298, 299)

1.3.5.1 *Novel compounds targeting GRP78*

Recently, a number of novel naturally occurring compounds have been identified from bacterial culture broths that can down-regulate GRP78. (-)-epigallocatechin gallate (EGCG), a major component of green tea, inhibits GRP78 activity and can increase etoposide-induced apoptosis in cancer cells. This is in addition to its activity on many other cellular targets.(300) Versipelostatin (VST) from *Streptomyces versipellis*,(301) Valinomycin from *Actinomycete*,(302) Verrucosidin from *Penicillium verrucosum* var. *Cyclopium*,(303) and Piericidin A from *Streptomyces* sp.(304) have all been shown to downregulate GRP78. By suppressing GRP78 promoter activity with subsequent reduced production of UPR transcriptional activators, these compounds are cytotoxic to 2-DG stressed cells in vitro.(302-304) In xenograft models, VST showed anticancer activity at well-tolerated doses, and was found to have a synergistic anti-tumour effect with cisplatin suggesting a role for such inhibitors of ER stress to eliminate otherwise drug-resistant, hypoglycaemic tumour cells.(301)

The utility of the GRP78 promoter in human cancer gene therapy has also been examined. GRP78 promoter-driven suicide gene therapy activated by photodynamic

therapy led to regression of sizable human breast cancer tumour in mouse xenograft models in combination with ganciclovir. The potential benefit to this therapy is that gene expression driven by the GRP78 promoter is minimal in most major organs and normal tissue.(305)

The presence of functional GRP78 on the cell surface of cancer cells provides a novel therapeutic target.(208, 213, 215, 217) Using a cell surface GRP78 binding peptide synthesized as a chimeric peptide with a proapoptotic sequence, tumour growth in human prostate cancer xenografts and murine breast isogenic tumours was suppressed.(214) Antibodies directed against the GRP78 COOH-terminal domain (anti-CTD antibody) on the cell surface can upregulate p53 activity and promote apoptosis.(306) Furthermore, anti-CTD antibody also downregulates the UPR and upregulates proapoptotic protein GADD153, and the apoptotic components, cleaved PARP-1 and Erdj5.(307) By contrast, patients who develop autoantibodies against the $\alpha 2M^*$ binding site on the NH₂-terminal domain of cell surface GRP78, show agonistic upregulation of GRP78 and its antiapoptotic properties, conveying a worse prognosis.(211, 214) GRP78 on the cell surface of proliferating endothelial cells and a variety of stressed tumour cells serves as a binding site for Kringle 5 (K5), an angiogenesis inhibitor. K5 is a human plasminogen proteolysed product that can inhibit angiogenesis by causing apoptosis of proliferating endothelial cells. Recombinant K5 shows high affinity binding to cell surface GRP78 and can produce apoptosis selectively in hypoxic tumour cells via enhanced activity of caspase-7.(308)

A disadvantage of these GRP78 targeting compounds is their lack of specificity and the unknown effects upon other pathways. A compound that specifically targets

GRP78 is likely to be a useful tool for probing UPR pathways and given the implication of the UPR in cancer growth and development, is likely to provide novel anticancer strategies.

1.3.5.2 Subtilase cytotoxin and EGF-SubA

Subtilase cytotoxin (SubAB) is a recently discovered member of the AB₅ toxin family which are important virulence factors for a number of notorious bacterial pathogens including Shiga toxins produced by Shiga toxigenic *Escherichia coli* (STEC) and *Shigella dysenteriae*, cholera toxin (Ctx) from *Vibrio cholera*, labile enterotoxins from enterotoxigenic *E.coli*, and pertussis toxin (Ptx) produced by *Bordetella pertussis*. It was discovered in a strain of *Escherichia coli* that caused an outbreak of haemolytic uraemic syndrome in Adelaide in 1998.(309) SubAB consists of a binding pentamer of B units and an active A subunit, similar to A and B subunits of Shiga toxins from strains of *E.coli*. The A subunit of SubAB, unlike Shiga toxin, is a subtilase-like serine protease that, when transported to the ER, selectively attacks GRP78. SubA kills cells by specifically cleaving GRP78 at a single amino acid point (Leu416 and Leu417) in an exposed loop that links two GRP78 domains: the ATPase and the substrate-binding domain.(310, 311) In contrast to the downregulation of GRP78 which does not significantly affect cell growth,(294) selective cleavage of GRP78 by SubA releases PERK, IRE1 and ATF6 leading to overwhelming activation of the UPR and subsequent apoptosis.(312, 313)

To reduce the potential for significant systemic side effects of administering SubA as a potential therapeutic agent, tumour targeting must be achieved. EGFR is frequently overexpressed on the surface of tumour cells making it an attractive drug target.

Ligand binding to EGFR leads to internalisation and trafficking. An engineered fusion protein, epidermal growth factor (EGF)-Sub A, EGF and Sub A, can produce significant inhibition of human breast and prostate tumour xenografts and can dramatically increase the sensitivity of cancer cells to thapsigargin, an ER stress inducing drug, and vice versa.(314) Investigation locally within the Boyd laboratory have also demonstrated that EGF-SubA is cytotoxic in laryngeal squamous carcinoma cells at picomolar concentrations, produces at least an additive effect when combined with cisplatin and may act as a radiosensitising agent. (M. Aslam, thesis). No evidence exists as to the effects of EGF-SubA in other solid tumours or cell line derivatives.

1.3.5.3 GRP78 and colorectal cancer

GRP78 remains relatively understudied in human colorectal cancer. In colon cancer, Xing and colleagues found GRP78 expression to be upregulated with progression along the normal tissue-adenoma-carcinoma sequence suggesting a role in malignant transformation.(315) Liu and colleagues also confirmed GRP78 expression to be elevated in tumour but found no correlation with clinicopathological variables.(316) GRP78 overexpression in colon cancer is also noted in the presence of other molecular chaperones (cytosolic chaperonin, GRP94, HSC70).(317)

Only one group has attempted to examine for any correlation between GRP78 expression and survival following surgery for colorectal cancer. Takahashi and colleagues analysed post-surgical survival of 262 patients with adenocarcinoma.(318) Interestingly, a survival advantage of nearly 10% was observed for patients with elevated GRP78 expression although this did not reach

statistical significance ($P=0.198$). The authors have not discussed this potentially important observation any further. This data suffers however as survival analysis did not take account of any adjuvant treatment received post-operatively and immunohistochemistry was performed using an antibody (sc-1050, Santa Cruz Biotechnology) which I believe to be non-specific for GRP78 as I will demonstrate in this thesis.

1.4 Aims and objectives

1.4.1 GRP78 expression as a predictive biomarker of response to adjuvant fluorouracil-based therapy in colorectal cancer

Colorectal cancer remains a major cause of mortality. At the molecular level, colorectal cancer is a heterogenous disease and this accounts for differences in survival and responsiveness to chemotherapy. As a result, many patients do not benefit from treatment, while others may require combination therapy as oppose to a single agent. Furthering the understanding of drug resistance in colorectal cancer by identifying prognostic and predictive biomarkers is vital. Although well characterised in vitro, the understanding of the influence of GRP78 in human cancers is very limited, with a paucity of data for colorectal cancer. Its relationship to drug sensitivity in vitro and its ease of detection by immunohistochemistry make GRP78 a potentially attractive biomarker. The aims of this project therefore were:

1. To identify a suitable antibody to detect GRP78 on a colorectal tissue microarray and optimise conditions for immunohistochemistry.
2. To examine if GRP78 expression correlated with malignancy and was predictive of survival or benefit from 5-FU based chemotherapy.
3. To determine how depleting endogenous GRP78 in vitro would alter the cellular response to 5-FU.

1.4.2 Pre-clinical investigation of GRP78 as a potential therapeutic target in colon cancer using a novel engineered fusion protein; EGF-SubA.

Limited data exist suggesting that EGF-SubA is highly toxic to several cancer cell lines and may reduce tumour growth in mouse xenograft models. Recent work in the Division of Surgery and Oncology, University of Liverpool indicates that EGF-SubA was toxic to head and neck squamous cell carcinoma (HNSCC) cell lines and toxicity correlated with cell surface EGFR expression. Furthermore, and of relevance to head and neck cancer, EGF-SubA showed evidence of synergy with cisplatin and potential as a radiosensitising agent.

To our knowledge, no data currently exist as to the effect of this agent in the setting of colorectal cancer. The aims of this project were:

1. To characterise the EGFR surface expression of a panel of colon cancer cell lines and correlate this with sensitivity to EGF-SubA.
2. To explore the mechanism of action of EGF-SubA with regard to its biological activity and pharmacokinetics, its inhibitory effects upon proliferation, and ability to induce apoptosis.
3. To evaluate the potential role of EGF-SubA in the setting of the contemporary adjuvant treatment of colorectal cancer.

2 Materials and Methods

All water used in the experiments described was >18M Ω resistance purity.

2.1 Immunohistochemistry

2.1.1 Initial Sample Preparation: Deparaffinization and rehydration

Formalin fixed, paraffin embedded tissue sections mounted on glass slides, provided by the Liverpool University Tissue Bank, were labelled by pencil and mounted in a slide rack. Slides were deparaffinised and rehydrated by placing two times in Xylene for 10 minutes followed by 30 seconds in each of the graded alcohol solutions moving from 100%, 95%, 90%, to 70%. Slides were then treated in a beaker of tap water for 5 minutes.

2.1.2 Blocking and antigen retrieval

Table 2.1-1: Composition of PBS/Tween

Concentration	Reagent	Source
0.065 M	Na ₂ HPO ₄ .2H ₂ O	Reidel-de Haen
0.015 M	NaH ₂ PO ₄ .2H ₂ O	Fluka
0.075 M	NaCl	GPR
0.1% v/v	Tween 20	Sigma

Table 2.1-2: Endogenous peroxidase blocking buffer

Concentration	Reagent	Source
99% v/v	Methanol	Fisher Scientific
1% v/v	H ₂ O ₂	

Endogenous peroxidase was blocked by placing in buffer (1% H₂O₂ in Methanol) for 12 minutes, then slides were washed in PBS/Tween-20 for 5 minutes. Any slides not

to be subjected to antigen retrieval were separated. Slides for antigen retrieval were boiled in 10 mM citric Acid buffer (pH6) in a microwave for 25 minutes then left to cool for 20 minutes. A further wash in PBS/Tween-20 was performed and slides transferred to a moistened chamber to air dry. Tissue was marked with a PAP pen to form a hydrophobic barrier.

2.1.3 Indirect immunostaining

Table 2.1-3: Primary antibodies used in this project for immunohistochemistry

Protein	Antibody	Source	Raised in	Working concentration
GRP78	sc-1050 (N20)	Santa Cruz Biotechnology	Goat polyclonal	1:100
GRP78	sc-13968 (H129)	Santa Cruz Biotechnology	Rabbit polyclonal	1:50

Table 2.1-4: Biotinylated secondary antibodies used in this project for immunohistochemistry

Target	Antibody	Source	Raised in	Working concentration
Anti-Rabbit	BA-1000	Vector Labs	Goat	1:200
Anti-Goat	BA-5000	Vector Labs	Rabbit	1:200

Slides were incubated at room temperature for 30 minutes in 2.5% normal serum (Vector Laboratories) of the species in which the secondary antibody was raised. As an example, for anti-GRP78 sc13968, 250 µl of goat serum was added to 10 ml Dulbecco's phosphate buffered saline (PBS) (Sigma) and mixed well. For anti-GRP78 (sc1050), 250 µl of rabbit serum was added to 10 ml PBS and mixed well. Approximately 100-200 µl of blocking serum is needed per slide.

The blocking solution is then removed carefully and the tissue then covered with the primary antibody diluted with blocking solution, covered and incubated at room temperature for 1 hour. Omission of the primary antibody is used as a negative control. It is important to avoid the edges of the tissue drying out while the antibody is applied.

Wash slides in PBS/Tween-20 for 5 minutes then incubate at room temperature for 30 minutes with diluted (1:200 in buffer containing 2.5% serum) biotinylated secondary antibody. Wash in PBS Tween-20 for 5 minutes. This washing step is very important to minimise background.

After blotting off excess PBS/Tween-20, slides were incubated in Vectastain ABC reagent (Vector Laboratories) for 30 minutes then washed again in PBS/Tween-20 for 5 minutes.

2.1.4 Developing and counterstaining

DAB is used as a chromogenic substrate. The antibodies were revealed by DAB SK-4100 peroxidase substrate kit (Vector Laboratories) at room temperature for 10 minutes in the dark. The reaction was stopped by rinsing in water for 5 minutes then slides were counterstained in haematoxylin for 45 seconds followed by a further rinse in running tap water until clear. Dehydrate samples with alcohol and xylene, 30 seconds in each alcohol solution moving from 70%, 90%, 95% and 100% then twice in Xylene for 5 minutes. Slides were then mounted with coverslips to view.

2.1.5 Imaging

Immunohistochemical images produced in this thesis were obtained from a Nikon Eclipse E800 upright microscope using MetaMorph® Imaging System Version 3.0 for Microsoft® Windows™ from Universal Imaging Corporation.

2.2 Cell Culture Techniques

All tissue culture work was performed using aseptic technique in a class II laminar flow tissue culture cabinet. Cells were routinely maintained in a 175 cm² tissue culture flask (nunc™) and subcultured every 48-72 hours. All media and solutions were pre-warmed in a 37⁰C water bath prior to use.

For all cell lines, base medium was RPMI-1640 (Sigma) supplemented with 10% foetal bovine serum (Sigma), Penicillin 100 U/ml and Streptomycin 100 µg/ml (Sigma).

2.2.1 Subculture

Cells were routinely passaged at 70-90% confluence. Culture medium was removed and discarded. Cells were then rinsed with 5 ml of sterile PBS to remove any traces of residual media containing trypsin inhibitor. The PBS was removed, then, 2 to 3 ml of Trypsin-EDTA solution (Sigma) was added evenly to cells, by gently tilting the flask to ensure coverage, and the flask incubated at 37⁰C until the cell layer had dispersed, as observed under an inverted microscope. The trypsin was then neutralised by adding an equal or greater volume (usually 6 to 8mls) of growth media. Cells were then aspirated by gentle pipetting to produce a single cell suspension and aliquoted as desired or replated at the desired concentration for each

cell line (typically 1:8). Flasks were then made up with growth media, normally 25 to 30 ml.

2.2.2 Preparation of frozen cell stocks

Cells were subcultured as described, then transferred to a 15 ml centrifuge tube (Falcon). Cells were centrifuged at 300g, 4⁰C for 5 minutes. After discarding the supernatant, the cell pellet was resuspended in freeze media (FBS/10% v.v. DMSO) at a concentration of c.2x10⁶ cell/ml. 1ml aliquots of cells were transferred to a 1.5 ml polypropylene cryotube (Eppendorf) and immediately placed in a -80⁰C freezer for 24hrs prior to long term storage in liquid nitrogen.

As required, frozen stocks were defrosted in a 37⁰C water bath and immediately transferred to pre-warmed culture media.

2.3 Cell lines

All cells were grown from existing frozen stocks held by the Division of Surgery and Oncology, University of Liverpool, unless otherwise stated. These stocks originated from the American Type Culture Collection (ATCC), Virginia, USA. The authenticity of all cell lines is regularly checked by DNA profiling.

2.3.1 HCT116 (p53 +/+, +/-, -/-)

HCT116 was initiated from an adult male patient with colon cancer. p53 status is documented as wild type. p53 heterozygous +/- and homozygous -/- contain a Δ N40 mutation on one or both alleles respectively.

2.3.2 LoVo

LoVo was initiated in 1971 from a fragment of a metastatic tumour nodule in the left supraclavicular region of a 56-year-old Caucasian male patient with a histologically proven diagnosis of adenocarcinoma of the colon.(319) The original tumour was Duke's C, Grade IV. p53 status documented as wild type.

2.3.3 SW480

SW480 was established from a primary Duke's B adenocarcinoma of the colon in a 50 year old Caucasian male in 1978.(320) The cells express elevated levels of p53 protein.

2.3.4 SW620

SW620 was isolated from the tissue of the same 51-year-old Caucasian male as was SW480, 1 year after initial colon resection. A recurrence of the malignancy resulted in a wide-spread metastasis from the colon to an abdominal mass. SW620 was initiated by A. Leibovitz, et al.,(320) from a lymph node in the same manner as was the primary adenocarcinoma from which SW480 was derived the previous year. Levels of p53 protein expression are elevated. Although the SW620 cell line is established from a later metastasis, it only has one p53 mutation whereas the tumour specimen cell line SW480 established one year previously carries two missense mutations.(321)

2.3.5 HT-29

HT-29 originated from a primary colon adenocarcinoma in a 44 year old Caucasian female in 1964. The cells express elevated levels of mutant p53.

2.4 siRNA knockdown

2.4.1 siRNA delivery

Short interfering RNA (siRNA) oligonucleotide knock-down experiments were performed in 6 well tissue culture plates (Corning, USA). Cells were harvested from their normal culture flask as described earlier and seeded aiming for a confluence of 30-50% on the day of transfection.

The siRNA was reconstituted in 1X siRNA buffer (diluted with RNase-free water from 5X siRNA buffers – Dharmacon) according to the manufacturer's directions to produce a 20 μ M stock. The siRNA oligonucleotides were used at a final concentration of 40 nM.

Lipofectamine™ 2000 (Invitrogen) was used as a transfection reagent. Lipofectamine is a cationic lipid that forms a positively charged liposome/siRNA complex that can fuse with the negatively charged cell membrane and then enter via endocytosis. Once inside the cell, the cationic lipids are believed to destabilise the endosome membrane thereby releasing the siRNA complex into the cytoplasm.(322) The siRNA molecules dissociate from the lipid complex and enter the nucleus becoming a part of the RNA-induced silencing complex (RISC). Guided by the antisense strand of the siRNA, RISC cleaves the target mRNA inhibiting its translation.(323)

On the day of transfection, usually 24hr after seeding, the cells were washed with sterile PBS and 1.5 ml of antibiotic free fresh media were applied per well. Opti-MEM®I Reduced Serum Media (Invitrogen) was pre-incubated to 37°C. For each transfection, the oligomer-Lipofectamine complex (500 µl/well) was prepared by initially producing two solutions. For each well of a 6 well plate, 4 µl of 20µM stock siRNA (80picomoles) is diluted in 246 µl Opti-MEM®I Reduced Serum Media (Invitrogen) to make up to 250 µl/well. This solution was vortexed gently and allowed to stand for 5 minutes. A further solution containing 4 µl/well Lipofectamine and 246 µl/well Opti-MEM is made up, tapped gently with a finger and left to stand for 5 minutes. After the 5 minute incubation period, the two solutions are combined, mixed gently and allowed to stand for 25 minutes. Transfection is performed by adding the oligomer-Lipofectamine complex evenly in a drop wise manner to each well then mixing gently by rocking the plate back and forth to avoid concentration of siRNA/Lipofectamine complexes in the well. The addition of the siRNA/Lipofectamine solution gives a final transfection volume of 2 ml and a final siRNA concentration of 40 nM. The cells were incubated in transfection reagent for 6 hours and then the media was replaced with growth media to minimise potential toxic effects due to prolonged exposure to the Lipofectamine reagent. All volumes of Lipofectamine, OptiMEM and siRNA should be multiplied by 1.5 to account for loss during pipetting. Due to the growth characteristics of the colon cancer cells, it was often necessary to split the cells 24hr after transfection and replate them in a larger 10cm culture dish prior to drug treatment. Analysis of knockdown efficiency was performed at the protein level by standard SDS-PAGE and western blotting.

2.4.2 siRNA oligonucleotides

The following siRNAs, obtained from Dharmacon were used:

2.4.2.1 *GRP78*

siGENOME D-008198-03:

Target sequence: 5'-CCA CCA AGA UGC UGA CAU U-3'

siGENOME D-008198-04:

Target sequence: 5'-GAA AGG AUG GUU AAU GAU G-3'

siGENOME D-008198-05:

Target sequence: 5'-CGA CUC GAA UUC CAA AGA U-3'

siGENOME D-008198-18:

Target sequence: 5'-CAG AUG AAG CUG UAG CGU A-3'

2.4.2.2 *Scrambled*

Target sequence: 5'-GGA CGC AUC CUU CUU AAU U-3'

2.5 Western Blotting

2.5.1 Cell lysate preparation

The desired cells were subcultured and harvested by trypsinisation and pelleted by centrifugation as described. Cell pellets were lysed on ice in SLIP in the presence of protease inhibitors: aprotinin (2 µg/ml), leupeptin (0.5 µg/ml), pepstatin A (1 µg/ml), soybean trypsin inhibitor (100 µg/ml) and phenylmethylsulfonyl fluoride (PMSF) (1 mM). The cell lysate was then centrifuged at 12,000g, 4°C for 10 minutes in an Eppendorf 5415R centrifuge, to remove cellular debris. The resulting supernatant was utilised and the pellet discarded.

Table 2.5-1: SLIP (Stuart Linn Immunoprecipitation) buffer

Concentration	Reagent	Source
50 mM	HEPES, Molecular Biology Grade, pH7.5	Calbiochem
150 mM	NaCl	VWR
10% (v/v)	Glycerol, ≥99.5%, A.C.S. reagent	Sigma
0.1% (v/v)	Triton X-100	Amersham Biosciences

Table 2.5-2: Protease inhibitor cocktail

Concentration	Reagent	Source
2 µg/ml	Aprotinin	Roche
0.5 µg/ml	Leupeptin	Roche
1 µg/ml	Pepstatin A	Roche
1 mM	Phenylmethylsulfonyl fluoride (PMSF)	Fluka

2.5.2 Bradford assay for determination of protein concentration in cellular extract

Bradford reagent (Bio-Rad) was diluted 1:5 in H₂O. A series of protein standards was prepared by adding bovine serum albumin (Sigma) in a concentration of 20 mg/ml to 1ml of SLIP lysis solution in a clean 1.5ml Eppendorf tube. 0.5ml was serially extracted and added to a further 1.5 ml Eppendorf containing 0.5 ml of dilute Bradford reagent resulting in prepared protein standards of 20, 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0 mg/ml. The optical density at 595 nm of each standard protein concentration was measured using a spectrophotometer (Eppendorf Biophotometer). A variance of <5 in the c.v. reading was deemed acceptable.

Table 2.5-3: Protein sample loading buffer (4X)

Concentration	Reagent	Source
250 mM	Tris, pH 6.8	Calbiochem
8% (v/v)	Sodium dodecyl sulphate (SDS)	BDH Prolabo
40% (v/v)	Glycerol, ≥99.5%, A.C.S. reagent	Sigma
1% (v/v)	B-mercaptoethanol	Sigma
4 mg/ml	Bromophenol blue	Sigma

Protein concentrations of each supernatant were determined by adding 2 µl of supernatant to 1ml of dilute Bradford reagent and measuring on the spectrophotometer. Samples were then adjusted to load 50 µg of protein/20 µl in 1X protein sample buffer. If protein yield was too high (>20 mg/ml) then a further 100 µl of SLIP lysis solution was used to dilute the supernatant and Bradford assay repeated for that sample. Samples were then stored at -80°C or prepared for western blot analysis.

2.5.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Table 2.5-4: Composition of SDS-PAGE gels (volume needed for 4 gels)

Reagent	Source	Seperating Gel			Stacking gel
		7.5%	10%	12%	
H ₂ O	-	5.42 ml	4.8 ml	4.3 ml	7.225 ml
40%(v/v) Acrylamide mix	BDH	1.87 ml	2.5 ml	3 ml	1.275 ml
1.5 M Tris-HCl (pH 8.8)	Calbiochem	2.5 ml	2.5 ml	2.5 ml	-
1 M Tris-HCl (pH 6.8)		-	-	-	1.25 ml
10% SDS	BDH	0.1 ml	0.1 ml	0.1 ml	0.1 ml
10%(w/v) Ammonium persulfate (APS)	Sigma	0.1 ml	0.1 ml	0.1 ml	0.1 ml
N,N,N',N'-tetramethylethylenediamine (TEMED)	BDH	0.008 ml	0.008 ml	0.008 ml	0.01 ml

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a Bio-Rad Mini-Protean III Cell Electrophoresis System. 0.75 mm glass plates were cleaned with 70% ethanol and mounted. Adequate positioning of the glass plates on the mount was confirmed by testing with H₂O before pouring the gels. First, a separating gel was poured between the plates to within 1.5 cm of the top, usually 4mls. The percentage acrylamide mix of the gel used depended upon the expected size of the target protein, with the 10% gel most commonly used. The gel was covered with 1ml of H₂O and left to polymerise for 1 hour before adding the stacking gel. The H₂O was poured off and the stacking gel poured to the top of the glass plates. Having carefully positioned a 10 well comb, the gel was left to set for 15 minutes. Once set, the comb was removed and the wells irrigated with H₂O. The gel and plates were positioned in the electrophoresis chamber and Tris-Glycine

buffer (25 mM Tris-HCl, 250 mM Glycine, 0.1% (v/v) SDS) poured to fill the central chamber and half fill the outer chamber (500 ml per chamber). The protein samples to be analysed were first boiled on a hot block at 99°C for 5 minutes then centrifuged (Eppendorf 5804R) at 14000, 4°C for 2 minutes. 50 µg (20 µl) of total protein in 1X sample buffer was added to each well and resolved alongside 15 µl of protein marker (New England Biolabs). Empty wells were loaded with 15 µl of 1X protein sample buffer. Proteins were separated by SDS-PAGE at 200 V for 45-60 minutes.

2.5.4 Protein Transfer to Hybond ECL Nitrocellulose Membrane

The proteins on the gel were transferred to a pre-cut (5x8 cm) Hybond ECL nitrocellulose membrane (Amersham Biosciences) using a Bio-Rad Mini Trans-Blot system. Sponges, membrane and pre-cut 3MM Whatman Chromatography paper were pre-soaked in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% (v/v) methanol) before arranging the gel and membrane within a sandwich of 3MM Whatman paper (10x7cm) and sponge inside a cassette. The cassette was loaded so that the gel was toward the negative electrode and the membrane towards the positive electrode. 100 V was applied across the cassette for one hour in a transfer tank filled with transfer buffer and containing an ice block to aid cooling.

When transfer was complete, the membrane was removed and stained with Ponceau S (0.2% (w/v) Ponceau S (Sigma), 5% (v/v) acetic acid (Merck)) for 1 minute to confirm equal protein transfer, washed in water and photographed. The membrane could be cut horizontally at this stage, at levels dependent upon which protein was being studied and guided by the protein marker as a guide. Membranes were then destained by several washes in PBS/Tween-20 and placed in 5% non-fat dry milk

(Bio-Rad) in PBS/Tween-20 overnight at 4⁰C, to block any non-specific protein binding sites.

2.5.5 Immunoblotting

Table 2.5-5: Primary antibodies used for immunoblotting

Protein	Antibody (clone number)	Source	Species raised in and clonality	Working concentration
Actin	sc-8432 (C-2)	Santa Cruz Biotechnology	Mouse monoclonal	3 µg/ml
GADD 153 (CHOP)	sc-7351 (B-3)	Santa Cruz Biotechnology	Mouse monoclonal	3 µg/ml
EGFR	sc-03 (1005)	Santa Cruz Biotechnology	Rabbit polyclonal	3 µg/ml
GRP78	sc-1050 (N20)	Santa Cruz Biotechnology	Goat polyclonal	3 µg/ml
GRP78	sc-13968 (H129)	Santa Cruz Biotechnology	Rabbit polyclonal	3 µg/ml
Hsp70 (HSP72)	SPA-810D	Cambridge Bioscience	Mouse monoclonal	3 µg/ml
LC3	M152-3 (4E12)	Medical and Biological Labs Co.	Mouse monoclonal	3 µg/ml
p53	Ab-6 (DO-1)	Oncogene-Merck	Mouse monoclonal	1 µg/ml
PARP	P76420	BD Transduction Labs.	Mouse monoclonal	3 µg/ml

Membranes were blocked in PBS-Tween-20 (0.1% v/v) containing non-fat dry milk (BioRad) (5% w/v) for 1 h at room temperature before incubation with primary antibodies (each at 3 µg/ml, except anti-p53 at 1 µg/ml). Membranes were washed three times for 15 min in PBS-Tween-20 to remove any non-specific binding of the

antibody before addition of HRP-conjugated secondary antibody for 1 h at room temperature. Membranes were washed as before.

Table 2.5-6: Horseradish peroxidase-linked secondary antibodies used for immunoblotting

Target	Antibody	Source	Raised in	Working concentration
Anti-Goat	305-036-003	Jackson ImmunoResearch Labs.	Rabbit	1:20000
Anti-Mouse	RPN4201	GE-Healthcare	Sheep	1:2500
Anti-Rabbit	NA934	GE-Healthcare	Donkey	1:5000

2.5.6 Enhanced chemilluminescence (ECL)

Excess PBS/Tween-20 was carefully blotted from the membrane, which was then incubated for 1 minute with Western Lightening Chemilluminescence Plus Reagent (Perker Elmer) made up of equal volumes of Enhanced Luminol Reagent and Oxidising Reagent (typically 2 to 3 mls of ECL Reagent required per membrane). The ECL reagent was then removed thoroughly and luminescence detected using a Kodak IM4000 image station.

2.6 MTT proliferation assay

Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be related to the number of viable cells.(324, 325) Mitochondrial dehydrogenases cleave the tetrazolium ring, yielding purple MTT formazan crystals which are insoluble in aqueous solutions. The crystals can be dissolved in dimethyl sulfoxide (DMSO, Sigma). The resulting purple solution is

spectrophotometrically measured at a certain wavelength (usually between 500 and 600nm). An increase in metabolically active cell numbers results in an increase in the amount of MTT formazan formed and an increase in absorbance. The MTT assay is reliable for the in vitro measurement of cell growth characteristics and cytotoxicity studies despite limitations influenced by the physiological state of cells and variance in mitochondrial dehydrogenase activity in different cell types.(326) It should be remembered that the MTT assay measures metabolic activity and therefore should be performed during an active cellular growth phase (viable cells do not necessarily equal metabolically active cells).

2.6.1 Cell Seeding

Cells were harvested when at 70-90% confluence from their standard culture flasks and transferred to a 50ml falcon tube containing an appropriate amount of warm media. Cell density was determined from the average of a minimum of 4 counts using a cell counter (ZTM Series COULTER COUNTER®, Beckman Coulter). The harvested cells were then diluted in warm standard growth media to a concentration of 15,000 cells/ml and seeded to a 96 well plate (nuncTM flat bottomed 96 microwell plate, Ref: 734-2097), adding 200µl of cells/well (3000 cells/well) using a multichannel pipette (Costar®). 4 wells per condition were seeded. Duplicate plates were seeded to allow measurements on consecutive days. Growth curves and cytotoxicity assays were typically performed over 5-7 days (one plate is required for each day including day 0). The 96 well plates were then incubated at 37°C, 5% CO₂, for 24 hours prior to any treatment to allow cells to adhere.

2.6.2 MTT assay

Following the desired incubation period, 20µl of MTT (Sigma) solution is added to each well and incubated for 3½ hours. After removal of the supernatant, taking care not to displace the formazan crystals at the bottom of each well, 100µl of DMSO was added to dissolve the formazan crystals, producing a violet solution. Absorbance was measured at 590 nm. Through an interface with a desktop computer, the data was stored and transferred to a Microsoft® Excel® 2010 program for analysis.

2.7 Flow cytometry

Flow cytometry was performed on a Becton Dickinson FACSCalibur™ flow cytometer and results were analysed using CellQuest software (Becton Dickinson).

2.7.1 Preparation of materials and buffers

Propidium iodide (Sigma P4170) stock solution was made up to 1 mg/ml in PBS and stored at 4°C in a foil wrapped 50ml Falcon tube to protect from the light.

RNAse (Sigma R6513) was made up to 10 mg/ml in 0.01 M sodium acetate (pH 5.2) then boiled at 100°C on a heated stirrer block in a glass bottle with the lid placed loosely for 15 minutes to inactivate DNase contaminants. This was allowed to cool then 1/10th volume of 1M Tris HCl (pH 7.4) was added. The stock solution was aliquoted into 1ml Eppendorf tubes and stored at -20°C.

Citrate buffer with spermine (100 ml) was prepared as follows. A 3.4 mM citrate solution (10% citric acid, 90% tri-sodium citrate) was created by adding 0.34 ml citric acid to 3.06 ml trisodium citrate (3.4 ml of 0.1 M citrate into 100ml = 3.4 mM). To this was added 0.1% TritonX-100 (Fluka) and 0.5 mg/ml spermine

tetrahydrochloride (Sigma S1141). At this stage, the solution was adjusted to pH 7, followed by the addition of 1% FBS and 50µg/ml RNAase.

2.7.2 Cell cycle analysis with Propidium iodide

Propidium iodide (PI) is an intercalating agent and a fluorescent molecule that binds to DNA by intercalating between the bases. PI can also bind to double stranded RNA (thus the requirement for adding RNAse to remove RNA). When excited with 488 nm wavelength light, it fluoresces red and can be detected with 562-588 nm band pass filter. Propidium iodide is used as a DNA stain to evaluate cell viability or DNA content by flow cytometry.

Propidium iodide is not membrane permeable so requires permeabilisation of the cells' plasma membranes by incubating them in a buffer solution containing a detergent such as Triton X-100, or by fixating them in ethanol.

2.7.2.1 Isolation of cells

Cells were harvested with media into a 15ml Falcon tube and pelleted by centrifugation at 170g for 5 minutes. The cells were then washed in cold PBS and centrifuged on two occasions. The PBS was carefully removed with care not to disturb the pelleted cells.

2.7.2.2 Fixation/Permeabilisation

The pellet was re-suspended to create a single cell suspension by flicking the falcon tube harshly with a finger. Ice-cold 70% Ethanol (stored at -20°C) was added drop wise to the cells while slowly vortexing constantly to prevent cell aggregation. A

total of 5 mls of Ethanol was added to each pellet and the cells left on ice for 1 hour. (If required, after fixing, the samples could be stored for up to 2 weeks in the fridge prior to PI staining and flow cytometric analysis).

2.7.2.3 Staining with Propidium iodide

Fixed cells were centrifuged at 170g for 5 minutes and washed twice in PBS/0.5% Tween-20 to remove residual ethanol, re-suspending after each wash. In-between washes, the cells were counted at the coulter counter to proceed with 1×10^6 cells in total. After pelleting the cells for the final time, remove PBS/Tween-20 and re-suspend the pellet in 100 μ l of RNase (10 mg/ml) and leave on ice for 5 minutes.

A solution containing 850 μ l of PBS and 50 μ l of PI (1 mg/ml) per sample was prepared. 900 μ l of staining solution was added to each sample, mixed gently and left on ice, protected from light, for 30 minutes. Samples were then analysed on the flow cytometer. Typically 10,000 events were recorded.

2.7.3 Bivariate cell cycle analysis with BrdU and Propidium Iodide

Bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU) is a synthetic nucleoside that is an analogue of thymidine. BrdU is incorporated into newly synthesized DNA of replicating cells (during S phase), substituting for thymidine during DNA replication. Antibodies specific for BrdU can then be used to detect the incorporated chemical, thus indicating cells that were actively replicating their DNA. Flow cytometry allows simultaneous measurement of incorporated BrdU as well as the DNA content on a single cell level. Binding of the antibody requires denaturation of the DNA using

acid.(327) The BrdU technique offers more specific information regarding cell cycle kinetics than PI staining is able to provide.

2.7.3.1 BrdU staining and isolation of cells

Cells were treated with 20 μ M (final concentration) of BrdU (from 1 mM stock, Sigma) for 60 minutes prior to harvesting media and cells into a 15 ml Falcon tube. Cells were centrifuged at 350 g for 5 minutes and washed in cold PBS, two times. Cell concentration was determined using the coulter counter and the protocol proceeded with 2 million cells per sample.

Citrate buffer with spermine was added (1 ml per million cells) to allow extraction of nucleii, the sample vortexed gently, then left on ice for 10 minutes. Subsequently, 10 ml of PBS was added and the sample centrifuged at 470 g for 5 minutes at 4°C. The pellet was washed in PBS/0.5% Tween-20 then centrifuged again. The PBS/Tween-20 was aspirated leaving a small volume (50 μ l) above the pellet.

2.7.3.2 Fixation

The tube containing the sample was tapped vigorously several times to turn the pellet into a fine suspension. 3 ml of ice cold 70% Ethanol was added dropwise while vortexing constantly at low speed. The samples were then placed on ice for 1 hour.

2.7.3.3 DNA denaturation

The nuclei were centrifuged at 470 g for 5 minutes at 4°C and the Ethanol aspirated. Each sample was tapped vigorously to re-suspend the pellet which was then washed

with PBS/0.5% Tween-20, vortexed briefly, then centrifuged again. The PBS/Tween was aspirated following which 50 µl of stock RNAase was added to each pellet.

The nuclei pellet was re-suspended by tapping and denatured by adding 1 ml of 2N HCL/0.5% TritonX-100 dropwise whilst constantly vortexing. The first few drops are added very slowly to prevent irreversible aggregation. The samples were incubated at room temperature for 30 minutes. This step denatures the DNA to facilitate binding of the primary antibody to BrdU.

The acid is neutralised by adding double volume of 0.1 M sodium tetraborate (pH8.5) and incubating on ice for 10 minutes. Samples were then washed twice in PBS/0.5% Tween-20/1% BSA and centrifuged at 470 g for 5 minutes at 4°C. PBS/Tween-20/BSA was aspirated leaving 80 µl above the pellet.

2.7.3.4 Immunofluorescent staining

20µl of anti-BrdU mouse monoclonal antibody (BD Bioscience, BD347580) were added to each sample and the pellet re-suspended by gently vortexing. After incubating at room temperature for 30 minutes, a further wash with PBS/0.5% Tween-20/1%BSA was performed, nuclei were pelleted at 470 g for 5 minutes at 4°C, and then the PBS/Tween-20/BSA aspirated leaving 100 µl above the pellet. 2.7 µl of FITC-conjugated goat anti-secondary antibody (Sigma, F2012) were added to each sample which was then re-suspended by gentle vortexing and then incubated in the dark for 30 minutes. The sample was washed again in PBS/0.5% Tween-20 and pelleted as before. The supernatant was aspirated and the sample tube tapped vigorously and vortexed to turn the pellet into a fine suspension. 1 ml of propidium

iodide (10 µg/ml) was added to each sample, vortexed and then incubated on ice for 30 minutes, protected from light, prior to analysing on the flow cytometer.

2.7.4 Annexin V apoptosis detection

During apoptosis, cells undergo a number of morphological changes, including surface changes, which eventually result in recognition and uptake by phagocytes. In apoptotic cells, loss of plasma asymmetry results in the membrane phospholipid phosphatidylserine (PS) being translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment.(328) Annexin V is a 35-36 kDa Ca^{2+} -dependent phospholipid-binding protein that has a high affinity for PS, and binds to cells with exposed PS. Annexin V may be conjugated to fluorochromes including FITC without loss of affinity and thus serves as a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis. Since externalization of PS occurs in the earlier stages of apoptosis, FITC Annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation.

FITC Annexin V staining precedes the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Therefore, staining with FITC Annexin V is typically used in conjunction with PI to evaluate sub populations at different stages of apoptosis. Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. For example, cells that are considered viable are FITC Annexin V and PI negative; cells that are in early apoptosis are FITC Annexin V positive and PI

negative; and cells that are in late apoptosis or already dead are both FITC Annexin V and PI positive.(329)

2.7.4.1 Preparation of controls

In order to set up the parameters on the flow cytometer and adjust for channel colour compensation, a positive control was created by inducing apoptosis in cells using staurosporine, a tyrosine kinase inhibitor. Staurosporine (LC Laboratories), made up in DMSO to stock 1 mM, was incubated with cells for 4 hours at a working concentration of 1 µg/ml.

Negative controls were generated by incubating cells with a comparable volume of DMSO.

Instrument settings used for the annexin V assay in this thesis are shown in the Appendix section 6.1.

2.7.4.2 Isolation of cells and staining

Cells and their media were harvested and centrifuged at 150 g for 5 minutes at 4°C (NB: floating cell populations within the media represent late stage apoptotic and necrotic cells and excluding these may compromise the results). The pellet was washed twice in cold PBS then resuspended in 1ml of cold 1X Aposcreen™ Annexin V binding buffer (1 part diluted in 9 parts H₂O from 10X stock, Southern Biotech, 10045-01) to a concentration of 1×10^6 to 1×10^7 cells/ml and kept on ice. 100 µl of each sample was aliquoted into a flow cytometer tube and 10 µl of Aposcreen™ Annexin V Fluorescein (FITC) conjugate (Southern Biotech, 10038-02) added. The samples were vortexed gently and left in ice for 15 minutes protected from the light.

380 µl of cold 1X binding buffer was then added to each sample followed by 10 µl of PI (50 µg/ml) and the samples analysed on the flow cytometer immediately. Results were obtained from 20,000 events.

2.7.5 Detection of EGFR membrane expression

Flow cytometry was used to detect surface expression of EGFR as opposed to total EGFR as measured by western blot.

Cells (1×10^6) were harvested and centrifuged at 400 g for 5 minutes at 4°C. Media was removed and the pellet resuspended in 1 ml of ice cold PBS/10% FBS. The sample was then incubated with 10 µl of Phycoerythrin-conjugated anti-EGFR antibody (ICR10, Abcam, ab27764) for 30 minutes protected from the light. The cells were then centrifuged at 400 g for 5 minutes at 4°C and washed three times in PBS/10%PBS. After the final wash, the cells were resuspended in PBS/10%FBS and taken for analysis whilst still protecting from the light.

2.8 Statistical analysis

Statview version 5.0 (SAS Institute Inc, Cary, North Carolina, USA) was used for statistical analysis. A P value of less than 0.05 was considered significant. Comparisons between groups were made using the non-parametric continuity corrected χ^2 test. Survival analyses were performed using overall 5-year survival, calculated as time elapsed from the date of diagnosis to censoring at last contact with patient or death. Patients who died within 30 days of surgery were excluded from all survival analysis. Kaplan-Meier curves were used to evaluate the effect of GRP78 expression on survival; P values were calculated using the logrank test. A univariate

Cox proportional hazard regression was performed to assess the statistical significance of each clinical or pathological factor in relation to survival. A multivariate Cox regression model was performed using a stepwise forward selection process informed by the Akaike information criterion or AIC,(330, 331) which is a statistical estimate of the trade-off between the likelihood of a model against its complexity, with a lower value indicating a better model. The likelihood ratio χ^2 statistic was also assessed as an indicator of the global goodness of fit of the Cox model. Thus, prediction bias due to over-fitting the model should be avoided.

3 GRP78 expression as a predictive biomarker of response to adjuvant fluorouracil-based therapy in colorectal cancer

3.1 Results

3.1.1 Antibody validation and optimisation for immunohistochemistry

In view of the mixed reports of GRP78 expression in different tumour types (see section 1.3.4), it was hypothesised that some of these differences may be due to antibody specificity. A literature search was performed to identify papers that used antibodies to human GRP78 in immunohistochemistry. The results are summarised in Table 3.1-1 below.

Table 3.1-1: Papers studying immunohistochemical elevated expression of GRP78

Tissue type	Reference	GRP78 antibody	Survival
Gastric	(277)	sc1050 (Santa Cruz Biotechnology)	Worse
Oesophagus	(287)	anti-GRP78 (abcam)	Improved
Breast	(296)	sc-1050 (Santa Cruz Biotechnology)	Not specified
	(332)	sc-13968 (Santa Cruz Biotechnology)	Not specified
Lung	(285)	Anti-GRP78 (Santa Cruz Biotechnology) Not specified	Improved
Prostate	(280)	sc-13968(Santa Cruz Biotechnology)	Worse
	(279)	sc-13968(Santa Cruz Biotechnology)	Worse
Head and Neck	(275)	sc-1050 (Santa Cruz Biotechnology)	Not specified
Melanoma	(333)	sc-13968(Santa Cruz Biotechnology)	Worse
Colon	(315)	sc-1050 (Santa Cruz Biotechnology)	Not specified
	(316)	Anti-GRP78 (Santa Cruz Biotechnology) Not specified	Not specified
	(318)	sc-1050 (Santa Cruz Biotechnology)	Improved
Liver	(334)	Anti-GRP78 (Santa Cruz Biotechnology) Not specified	Not specified
Neuroblastoma	(286)	Anti-GRP78 (Santa Cruz Biotechnology) Not specified	Improved

Two antibodies used in published studies of GRP78 expression by immunohistochemical analysis (Santa Cruz Biotechnology antibodies to GRP78 sc-1050 and sc-13968) were selected for pre-validation. Cells were cultured for 24 hours in normal media or normal media supplemented with 1nM EGF-SubA (Sibtech. Inc.) or vehicle control. EGF-SubA (discussed in section 1.3.5.2) contains a fusion of EGF (which provides targeting to EGFR expressing cells) and the A subunit of a bacterial AB₅-type endotoxin with subtilisin-like proteolytic activity which has only one known substrate: GRP78 and cleaves a di-leucine motif (Leu₄₁₆-Leu₄₁₇) in an exposed loop that links two GRP78 domains: the ATPase and the substrate-binding domain. Thus treating EGFR positive cells with EGF-SubA enables confirmation of the identity of c. 78kDa bands obtained by western blotting with antibodies to GRP78.

Whereas the sc-1050 antibody () shows non-specific staining with a band running in advance of the 78kDa band that is not abolished by EGF-SubA treatment and we suspect is HSP72 (GRP78 shares 85% identity with HSP72), the sc-13938 antibody (Figure 3.1.1-2) shows minimal non-specific staining recognising a single protein band that is almost completely abolished following treatment with EGF-SubA inferring suitability for use in IHC.

Also note that sc-1050 with epitope mapping at the N-terminus of GRP78 detects the cleaved N-terminus 44 kDa fragment whereas sc-13968 with epitope mapping the C-terminus of GRP78 detects the cleaved C-terminus 28 kDa fragment following exposure to EGF-SubA.

In order to confirm if the additional band seen with sc-1050 is HSP72, SW480 cells were cultured for 24 hours in normal media or media supplemented with

Geldanamycin (LC Laboratories, NSC-122-750) with or without 1 nM EGF-SubA or vehicle control. Geldanamycin is a HSP90 inhibitor that is known to up-regulate HSP72.(335) Geldanamycin treatment resulted in increased expression of HSP72 (and GRP78 to a lesser degree) on western blot (see lanes 2 and 3) and this signal co-migrated with the band detected using the anti-GRP78 antibody sc-1050 (lanes 5 and 6, Figure 3.1.1-3). Although HSP72 closely resembles GRP78 it lacks the di-leucine motif (Leu₄₁₆-Leu₄₁₇) cleavage site and is therefore not subject to cleavage by EGF-SubA. In vitro, high doses of purified SubA are unable to cleave even the most closely related chaperones of the HSP70 family.(311) Importantly, it was observed that the band detected with sc-1050 is not degraded in the presence of EGF-SubA (lane 6, Figure 3.1.1-3).

It can thus be concluded that the sc-1050 antibody recognises HSP72 at higher levels in colorectal cells than GRP78 and this antibody clearly is not appropriate for use in IHC where subtleties of protein mass cannot be discriminated. Since the anti-GRP78 antibody sc-13968 predominantly detects only GRP78 we proceeded to use this to investigate GRP78 expression in our cohort of CRC samples.

Initial trial staining of paraffin-embedded formalin-fixed tissue sections, using the method described in section 0, was performed using sc-13968 anti-GRP78 antibody at varying concentrations from 1:50, 1:100 and 1:200 to identify the optimal concentration to use on the tissue microarray. At concentrations of 1:100 and 1:200, GRP78 staining was too weak for accurate interpretation whereas at 1:50, good cytoplasmic staining was evident without excessive non-specific staining so this was used as the final working concentration. GRP78 expression was detected in the

cytoplasm of all carcinoma sections with sparing of the nucleus as shown in Figure 3.1.1-4.

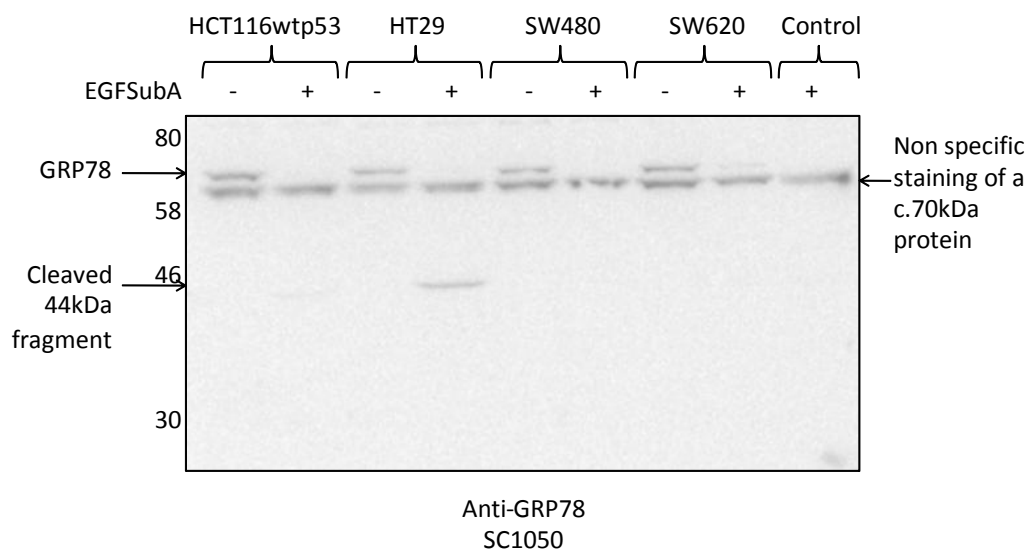


Figure 3.1.1-1: Western blot demonstrating immunostaining for GRP78 with sc-1050.

Cells were incubated with 1 nM EGF-SubA (SibTech) or vehicle control for 24 hr then harvested. Cell lysate from a head and neck cell line with confirmed GRP78 expression was used as a control. Cell lysates (50 µg per lane) were subject to western blot using goat polyclonal anti-GRP78 antibody (sc-1050, Santa Cruz) that recognises GRP78 and the 44 kDa cleaved GRP78 fragment. A further band c.70 kDa that is not subject to cleavage by EGF-SubA is also noted with this antibody.

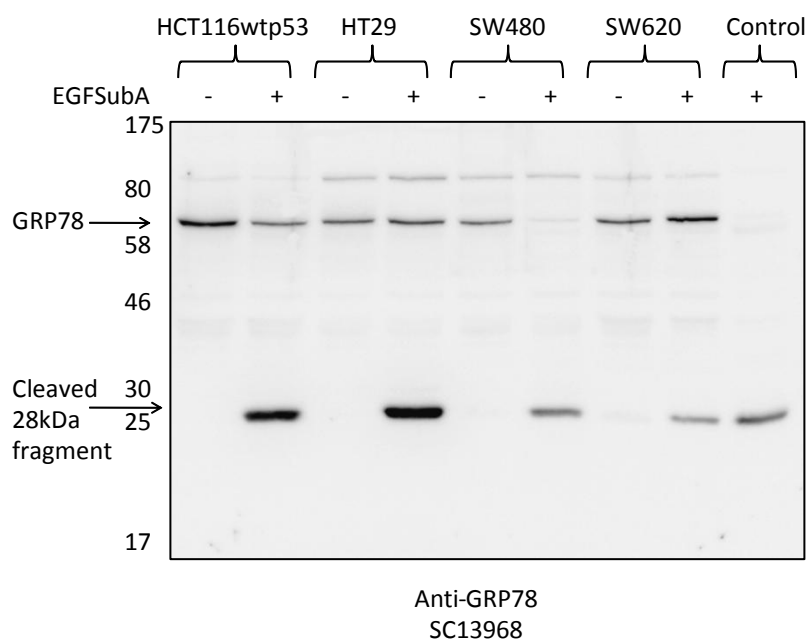


Figure 3.1.1-2: Western blot demonstrating immunostaining for GRP78 with sc-13968

Performed essentially as in but using the rabbit polyclonal anti-GRP78 antibody (sc-13968, Santa Cruz) that recognises GRP78 and the 28 kDa cleaved fragment. Cells were incubated with 1 nM EGF-SubA (SibTech) or vehicle control for 24 hr then harvested. Cell lysate from a head and neck cell line with confirmed GRP78 expression was used as a control. Cell lysates (50 µg per lane) were subject to western blot using rabbit polyclonal anti-GRP78 antibody

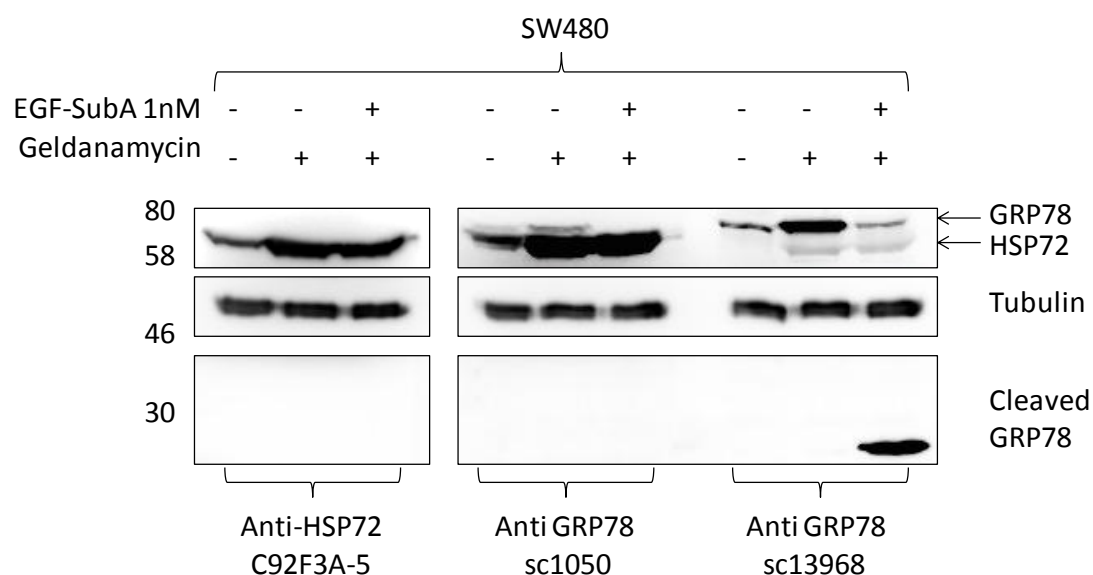


Figure 3.1.1-3: Western blot comparing detection of GRP78 using two different monoclonal antibodies; sc-1050 and sc-13968.

SW480 cells were incubated with either EGF-SubA 1 nM (SibTech) or Geldanamycin 10 μ M (LC Labs), a combination of these, or vehicle control for 24 hr. Cell lysates were subjected to western blot using antibodies to GRP78 (sc-13968 and sc-1050, Santa Cruz) and mouse monoclonal antibody to HSP72 (Cambridge Bioscience). Tubulin was used as a loading control.

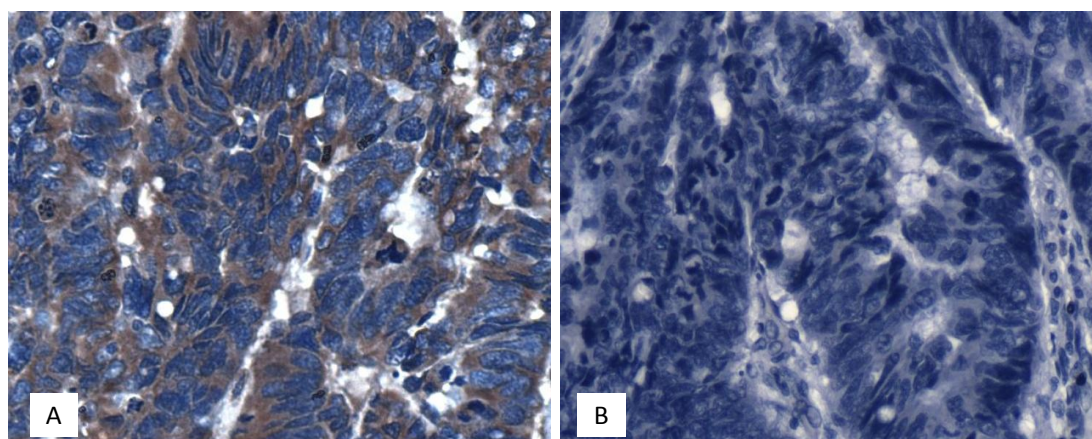


Figure 3.1.1-4: Representative sections of colon cancer stained with an antibody to GRP78 demonstrating cytoplasmic immunoreactivity with nuclear sparing.

FFPE sections of colon cancer were subject to immunohistochemistry using anti-GRP78 antibody sc-13968 (Santa Cruz). Original magnification x40. (A) Colon carcinoma section stained using sc13968 anti-GRP78 antibody. (B) Colon carcinoma stained without primary antibody as a control.

Following discussion with Dr Fiona Campbell (Consultant Pathologist) and Mr Andy Dodson (Dept. of Pathology) after initial trial staining on tissues section, it was suggested that the DAB time (see section 2.1.4) could be slightly prolonged without causing excessive non-specific staining and that excessive haematoxylin counterstaining should be avoided as this can mask areas of weak DAB staining. 3, 3' Diaminobenzidine (DAB) is a widely used chromogen for immunohistochemical staining and immunoblotting. In the presence of horseradish peroxidase and hydrogen peroxide, DAB oxidatively polymerizes to a brown polymer that is insoluble in alcohol and xylene. Due to the kinetics of DAB staining, the DAB time can be extended to some extent without causing significant non-specific staining.

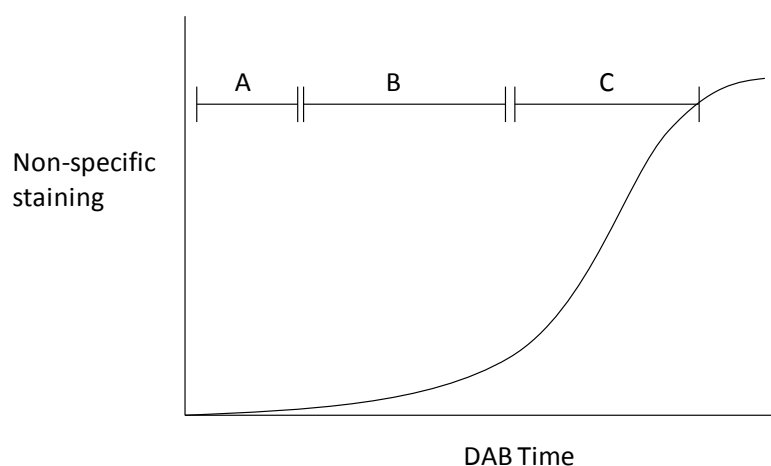


Figure 3.1.1-5: Illustration of the effect of DAB time on non-specific staining.

A, insufficient chromogenic staining; B, optimal staining; C, risk of non-specific staining. (Personal communication from Mr Andy Dodson)

Following these modifications, a tissue microarray was examined for GRP78 expression.

3.1.2 Patients

Appropriate ethical approval for use of this material for my research was obtained from Liverpool Adults Research Ethics Committee (REC 04/Q1505/125). Written consent for procurement and storage of tissue samples was sought before surgery after a full written and verbal explanation. Three hundred and ninety-six (194 colon, 202 rectal) consented patients who underwent surgery for colorectal cancer at the Royal Liverpool University Hospital, UK, between 1993 and 2003 were studied. Overall 5 year survival for this cohort was 46%. 114 patients (29%) received fluoropyrimidine-based adjuvant chemotherapy delivered at Clatterbridge Centre for Oncology NHS Foundation Trust, Wirral, UK. Of these, five patients with stage II disease were randomised to receive chemotherapy according to the QUASAR protocol (six 5-day courses every 4 weeks or as 30 once-weekly courses of intravenous (i.v.) fluorouracil (FU) (370 mg/m²) with high-dose (175 mg) L-folinic acid (FA) or low-dose (25 mg) FA),(83) and two patients were randomised to receive additional oxaliplatin as part of the MOSAIC trial (a 2-hour infusion of 200 mg of leucovorin per square meter of body-surface area followed by a bolus of FU (400 mg/m²) and then a 22-hour infusion of FU (600 mg/m²) given on 2 consecutive days every 14 days, for 12 cycles plus a two-hour infusion of oxaliplatin (85 mg/m²) on day 1, given simultaneously with leucovorin).(92) A further two patients received additional irinotecan (Irinotecan 180 mg/m² i.v.) for metastatic disease. The remaining patients received treatment according to local protocol which was i.v. FU and FA as per QUASAR or a modified de Gramont regimen (fixed dose leucovorin 350 mg (or 175 mg) as a 2-hour i.v. infusion; then i.v. FU bolus over 5 min; then 46-hour FU infusion, repeated every 14 days).(336) Decisions regarding adjuvant

chemotherapy were determined by patient choice, performance status and co-morbidities. 41 patients received further chemotherapy for relapse using additional agents such as irinotecan, oxaliplatin, and mitomycin C. Short course pre-operative radiotherapy was given to 13% with rectal cancer as part of the CR07 trial (25 Gy administered in 5 fractions over 1 week followed by surgery within 1 week)(111) and 15% received long course radiotherapy (45 Gy in 25 daily fractions over 5 weeks followed by surgery after 6-10 weeks). Tumours were staged using the TNM method (337). Median follow up was 49.5 months (range 0.23-146.5).

3.1.3 The colorectal tissue microarray

In order to examine whether the expression of GRP78 in colorectal tumours affects patients survival and response to adjuvant chemotherapy, I made use of a large colorectal tissue microarray (TMA) that exists locally due, in a large part, to the efforts of Ms Elizabeth Tweedle. A database with matched clinicopathological data was made available by Ms Tweedle including mortality data obtained from the North West Cancer Intelligence Service, Liverpool. Further data regarding chemotherapy, obtained from Clatterbridge Centre for Oncology, Wirral, was kindly provided by Mr Chin Wee Ang.

Tissue microarrays were created from 482 formalin-fixed paraffin-embedded primary tumours obtained from the Liverpool Tissue Bank Research Centre, University of Liverpool, and include two to six representative cores from each carcinoma and 16 cores each of normal kidney, liver, and testis which served as internal control tissues. The cores were arranged over 7 TMA's which were stained

simultaneously using the same stock of anti-GRP78 antibody (sc-13968) as used in the optimisation experiments.

Some patients were excluded from analysis due to cores being lost from the slides during processing or cores were too fragmented to interpret. Of the original 482 patients, acceptable cores and full data were available for 396 patients.

3.1.4 Scoring the tissue microarray

A review of immunohistochemical studies in human cancers reveals no standard scoring system of immunoreactivity of GRP78. A number of semi-quantitative scoring systems have been described with results demonstrating statistically significant correlations with clinical outcome. These consist of scoring systems based upon intensity of immunoreactivity (negative, weak, moderate and strong in most cases), and the percentage of cells with reactivity (0-100%). In the majority of studies, a final score is awarded based upon either the sum of intensity and percentage reactivity or their factor.(277, 280, 285-287, 316, 333, 334)

The integral control tissues of testis, normal kidney, and liver meant that each TMA essentially had a range of positive controls with testis staining weakly, liver staining strongly (not surprising given that the major function of hepatocytes is protein synthesis), and kidney showed intermediate staining. A row of control tissue is highlighted in Figure 3.1.4-1 above.

GRP78 immunoreactivity was seen ubiquitously in the cytoplasm of colonic carcinoma cells, and all tumour cells in a given patient stained equally. Minimal staining was evident in stromal tissue. Staining was categorised semi-quantitatively

using a scale of 0-3: 0 = negative, 1 = weak positive, 2 = intermediate positive and 3 = strongly positive (Figure 3.1.4-2).

The addition of a percentage positivity score was omitted in view of the uniform pattern of staining seen among tumour cells. The highest score from duplicate cores on the microarray was accepted for analysis. For data analysis, GRP78 expression was dichotomised based upon the mean score from the whole cohort (1.5) into either low expression (intensity 0 or 1), or high expression (intensity 2 or 3). Scoring was performed by a gastrointestinal histopathologist (Dr Fiona Campbell) who had no knowledge of the clinicopathological data of the patients.

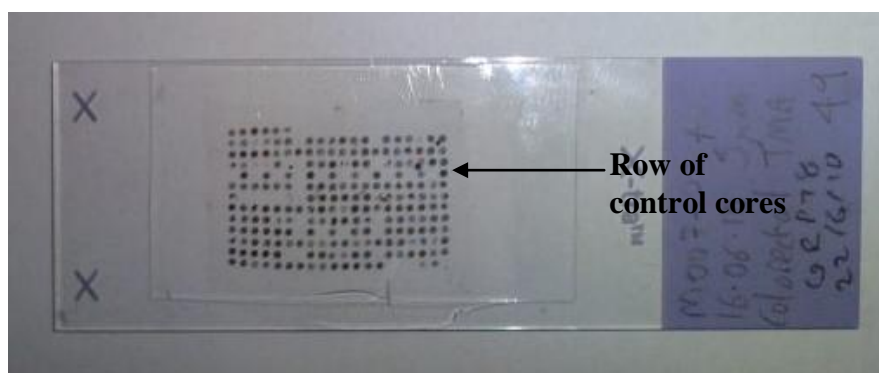


Figure 3.1.4-1: A section of the colorectal tissue microarray.

An example of one of the slides holding a section of the colorectal tissue microarray following staining and fixation. From this picture it is possible to identify differential staining of the individual cores of tissue.

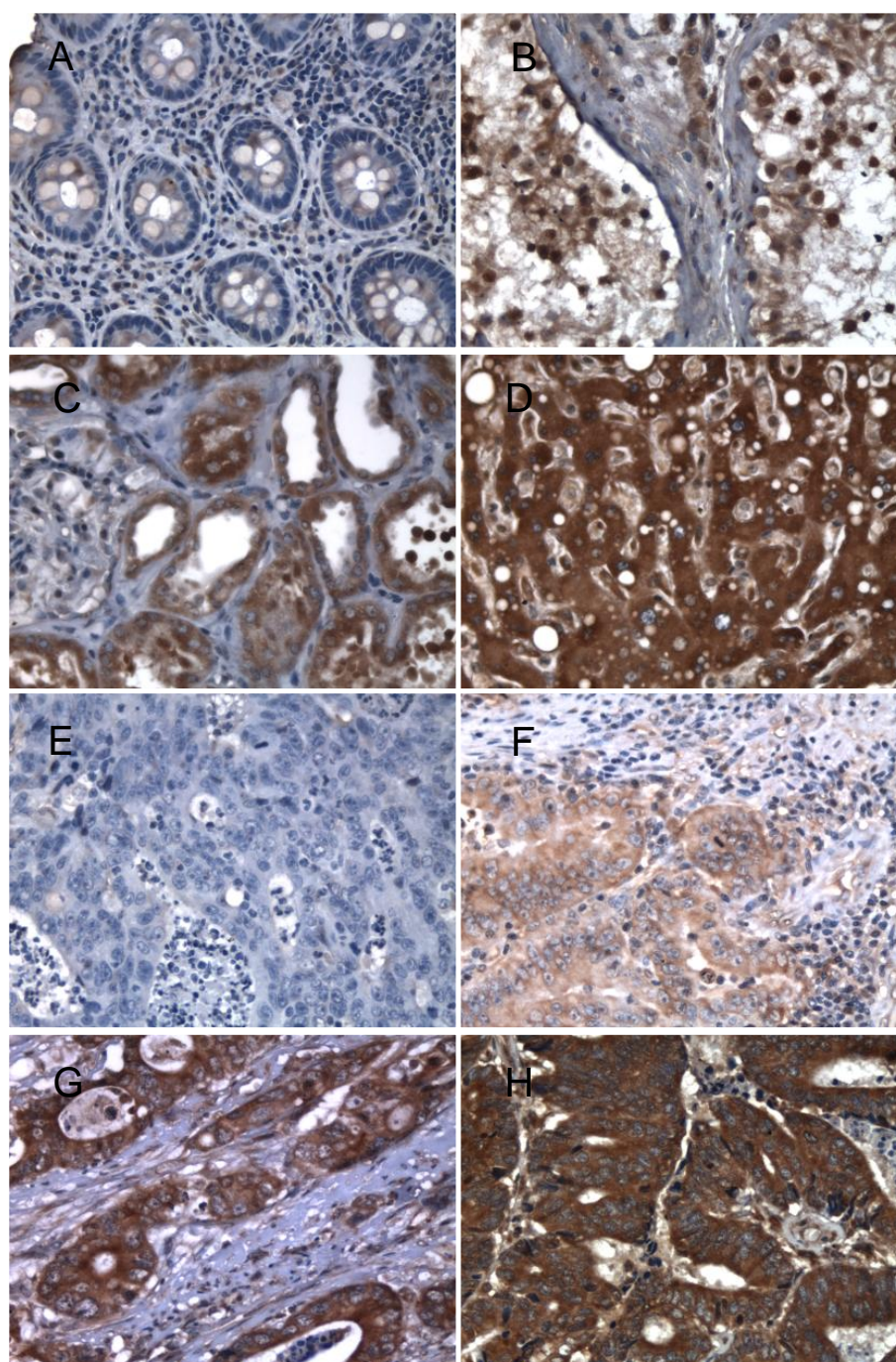


Figure 3.1.4-2: Immunohistochemical analysis of GRP78 expression in representative samples.

The TMA was stained using the rabbit polyclonal antibody specific for GRP78 (sc13968, Santa Cruz). This figure illustrates magnified views of representative cores from TMA. Control tissues (A) normal colon, (B) testis, (C) kidney, and (D) liver, gave a range of staining intensity for each section of the TMA; and colorectal cancer samples demonstrating (E) no staining=0, (F) weak staining=1, (G) moderate staining=2, and (H) strong staining=3. Original magnification x40.

3.1.5 GRP78 expression in colorectal cancer

Of 41 cases where matched normal colonic epithelium was available to score, GRP78 expression was significantly elevated (intensity 2 or 3) in 16 of 41 cancers vs. 1 of 41 matched normal colonic epithelium ($P<0.001$, **Error! Reference source not found.**).

Table 3.1-2:GRP78 expression in 41 patient-matched normal colon tissue and colonic adenocarcinoma

Tissue type (n=41)	Staining intensity				P
	0	1	2	3	
Normal tissue	7	33	1	0	P<0.001
Adenocarcinoma	0	25	14	2	

Data are number of patients. χ^2 test was used for comparison of variables.

3.1.6 Correlation of GRP78 with clinicopathological parameters

In keeping with a previous study,(316) no correlation could be found between GRP78 expression and gender, age, nodal status (pN stage), differentiation, tumour size, resection margin status, or administration of preoperative radiotherapy. There was a significant association between GRP78 expression and depth of invasion (pT, $P=0.029$) and stage ($P=0.032$) as shown in Table 3.1-3. This effect was largely due to GRP78 expression in rectal cancer and not colon cancer as shown in Table 3.1-4 and Table 3.1-5. Also of note is the observation that the administration of preoperative radiotherapy to rectal cancers did not lead to any difference in GRP78 expression.

Table 3.1-3: Association between GRP78 expression and clinicopathological parameters in 396 patients with colorectal cancer treated by surgery

Characteristic			GRP78 High		GRP78 Low		P-Value
	<i>n</i> =396	%	<i>n</i> =162	%	<i>n</i> =234	%	
Gender							
Male	248	62.6	102	63.0	146	62.4	0.964
Female	148	37.4	60	37.0	88	37.6	
Age							
<68 yrs	178	45	69	42.6	109	46.6	0.433
68yrs≤	218	55	93	57.4	125	53.4	
pT							
T1	19	4.8	7	4.3	12	5.1	0.029
T2	59	14.9	16	9.9	43	18.4	
T3	265	66.9	110	67.9	155	66.2	
T4	53	13.4	29	17.9	24	10.3	
pN							
N0	222	56.1	82	50.6	140	59.8	0.19
N1	99	25.0	46	28.4	53	22.6	
N2	75	18.9	34	21.0	41	17.5	
Stage							
I	60	15.2	16	9.9	44	18.8	0.032
II	162	40.9	66	40.7	96	41.0	
III	174	43.9	80	49.4	94	40.2	
Differentiation							
Well	10	2.5	3	1.9	7	3	0.755
Moderate	354	89.4	147	90.7	207	88.5	
Poor	28	7.1	12	7.4	16	6.8	
Unrecorded	4	1.0	0	0	4	1.7	
Tumour size							
<60mm	275	69.4	115	71.0	160	68.4	0.579
>60mm	121	30.6	47	29.0	74	31.6	
Resection margin							
Clear	351	88.6	138	85.2	213	91.0	0.099
Involved	44	11.1	23	14.2	21	9.0	
Unrecorded	1	0.3	1	0.6	0	0	
Adjuvant chemotherapy							
No	282	71.2	110	67.9	172	73.5	0.226
Yes	114	28.2	52	32.1	62	26.5	

Stage I, pT1-2/pN0; Stage II, pT3-4/pN0, Stage III, pT1-4/pN+

Table 3.1-4: Association between GRP78 expression and clinicopathological parameters in colon cancers

Colon Cancers							
Characteristic			GRP78 High		GRP78 Low		P-Value
	n=194	%	n=83	%	n=111	%	
Gender							
Male	101	52.1	44	53	57	51.4	0.819
Female	93	47.9	39	47	54	48.6	
Age							
<68 yrs	79	40.1	35	42.2	44	40	0.723
68yrs≤	115	59.9	48	57.8	67	60	
pT							
T1	12	6.2	5	6	7	6.3	0.533
T2	18	9.3	5	6	13	11.7	
T3	127	65.5	55	66.3	72	64.9	
T4	37	19.1	18	21.7	19	17.1	
pN							
N0	114	58.8	47	56.6	67	60.4	0.66
N1	43	22.2	21	25.3	22	19.8	
N2	37	19.1	15	18.1	22	19.8	
Stage							
I	24	12.4	10	12	14	12.6	0.871
II	90	46.4	37	44.6	53	47.7	
III	80	41.2	36	43.4	44	39.6	
Differentiation							
Well	4	2.1	1	1.2	3	2.7	0.745
Moderate	175	90.2	76	91.6	99	89.2	
Poor	13	6.7	6	7.2	7	6.3	
Unrecorded	2	1.0	0	0	2	1.8	
Tumour size							
<60mm	125	64.4	55	66.3	70	63.1	0.649
>60mm	69	35.6	28	33.7	41	36.9	
Resection margin							
Clear	179	92.3	74	89.2	105	94.6	0.249
Involved	14	7.2	8	9.6	6	5.4	
Unrecorded	1	0.5	1	1.2	0	0	
Adjuvant chemotherapy							
No	147	75.8	60	72.3	87	78.4	0.327
Yes	47	24.2	23	27.7	24	21.6	

Table 3.1-5: Association between GRP78 expression and clinicopathological parameters in rectal cancers

Rectal Cancers							
Characteristic			GRP78 High		GRP78 Low		P-Value
	n=202	%	n=79	%	n=123	%	
Gender							
Male	147	72.8	58	73.4	89	72.4	0.869
Female	55	27.2	21	26.6	34	27.6	
Age							
<68 yrs	99	49	34	43	65	52.8	0.174
68yrs≤	103	51	45	57	58	47.2	
pT							
T1	7	3.5	2	2.5	5	4.1	0.031
T2	41	20.3	11	13.9	30	24.4	
T3	138	68.3	55	69.6	83	67.5	
T4	16	7.9	11	13.9	5	4.1	
pN							
N0	108	53.5	35	44.3	73	59.3	0.098
N1	56	27.7	25	31.6	31	25.2	
N2	38	18.8	19	24.1	19	15.4	
Stage							
I	36	17.8	6	7.6	30	24.4	0.007
II	72	35.6	29	36.7	43	35.0	
III	94	46.5	44	55.7	50	40.7	
Differentiation							
Well	6	3	2	2.5	4	3.3	0.952
Moderate	179	88.6	71	89.9	108	87.8	
Poor	15	7.4	6	7.6	9	7.3	
Unrecorded	2	1.0	0	0	2	1.6	
Tumour size							
<60mm	150	74.3	60	75.9	90	73.2	0.659
>60mm	52	25.7	19	24.1	33	26.8	
Resection margin							
Clear	172	85.1	64	81.0	108	87.8	0.185
Involved	30	14.9	15	19.0	15	12.2	
Unrecorded	0	0	0	0	0	0	
Adjuvant chemotherapy							
No	135	66.8	50	63.3	85	69.1	0.392
Yes	67	33.2	29	36.7	38	30.9	
Neoadjuvant radiation							
No	143	70.8	55	69.6	88	71.5	0.769
Yes	59	29.2	24	30.4	35	28.5	

3.1.7 Survival and pathological factors

The overall 5-year survival for this cohort was 47% (Figure 3.1.7-1). This is in keeping with national figures from the late 1990's that quote five-year survival of 47% for colon cancer in both sexes, and 49% and 51% for rectal cancer in men and women respectively.(6)

As expected, survival was worse with increasing stage ($P<0.001$), presence of nodal metastasis ($P<0.001$), incomplete (R1) excision of the tumour ($P<0.002$), and poor differentiation ($P=0.023$).

The survival for patients with stage III disease is within the range of figures commonly quoted of between 27% and 60% (48) depending upon depth of invasion and degree of nodal involvement, however, the survival of patients with stage I and stage II is comparatively low. There are several possible explanations for this. As well as there being fewer stage I cancers than II or III, subgroup analysis reveals a difference in the mean age of patients at time of diagnosis between cancer stages; 69.9yrs (+/- 8.6yrs) for stage I, 68.5yrs (+/- 10.4yrs) for stage II, and 66.5yrs (+/- 11.8) for stage III disease. The older age of the stage I subgroup may in part contribute to the observed poor survival. Furthermore, and probably of more significance, the Mersey region has some of the lowest life expectancy (76yrs for men and 81yrs for women), (338) and worst cancer survival figures in the whole of England and Wales, both during the study period and to the present day.(339) The relatively low proportion of patients with stage I disease would suggest that patients in our region generally have more advanced disease at presentation. Evidence to support this comes from a European study (EUROCARE high resolution study on colorectal cancer)(340) that examined data from 11 European regions between 1988

and 1991, and reported that, in addition to one of the worst 3 yr survival rates in the regions studied (44% compared with highest of 59%), Mersey also had a proportionately higher number of Dukes C and D, and lower number of Dukes A and B cases during this period.

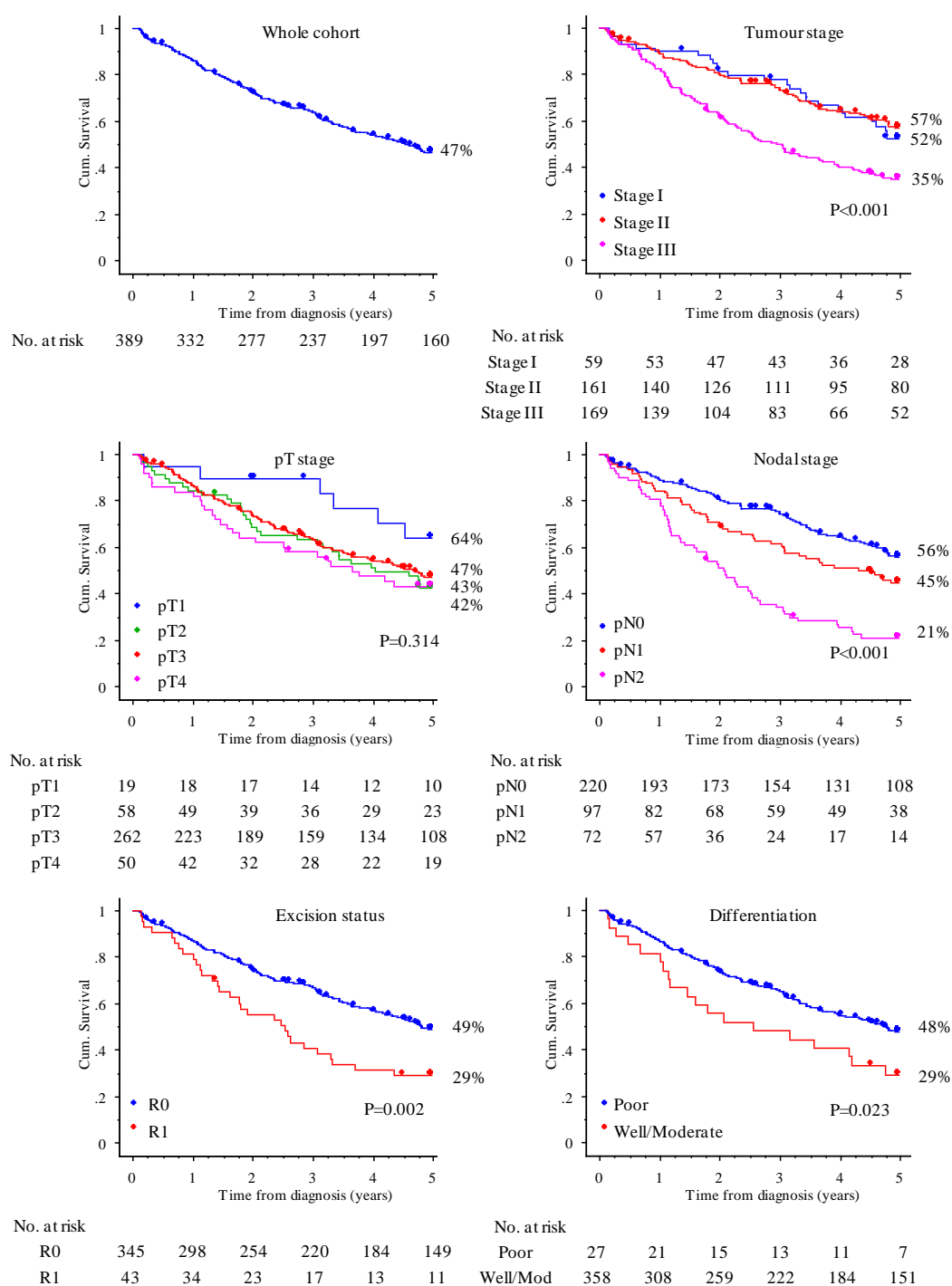


Figure 3.1.7-1: Kaplan-Meier plots demonstrating overall 5yr survival for the whole cohort (excluding those who died within 30 days of surgery) stratified by pathological variables.

3.1.8 GRP78 expression, survival and benefit from adjuvant chemotherapy

Better overall five-year survival was associated with elevated GRP78 expression in the whole cohort (53% v 42%; $P=0.036$, Figure 3.1.8-1A). Patients with stage II cancers treated by surgery alone with high GRP78 expression had improved survival (71% v 50%; $P=0.032$, Figure 3.1.8-1B). Insufficient numbers of stage II patients received adjuvant chemotherapy ($n=32$) to perform meaningful analysis of the influence of GRP78 expression.

Eighty patients with stage III cancer (46 rectal and 34 colon cancers) received 5-FU based adjuvant chemotherapy with a survival benefit of 10% at 5 years (40% v 30%; $P=0.15$, Figure 3.1.8-2). Of all patients who received adjuvant chemotherapy, elevated GRP78 predicted a better overall survival than low GRP78 (56% v 32%; $P=0.008$, Figure 3.1.8-2). As Figure 3.1.8-3 clearly demonstrates, elevated GRP78 predicts significant benefit from adjuvant chemotherapy (52% v 28%; $P=0.026$), whereas patients with low GRP78 failed to benefit (28% v 32%; $P=0.805$). Overall 5yr survival of stage III patients treated by surgery alone was not significantly different between high and low GRP78 expression (28% v 30%), however median survival was better by 7 months in the elevated GRP78 compared with the low GRP78 group (2.668 v 2.094 yrs, data not shown).

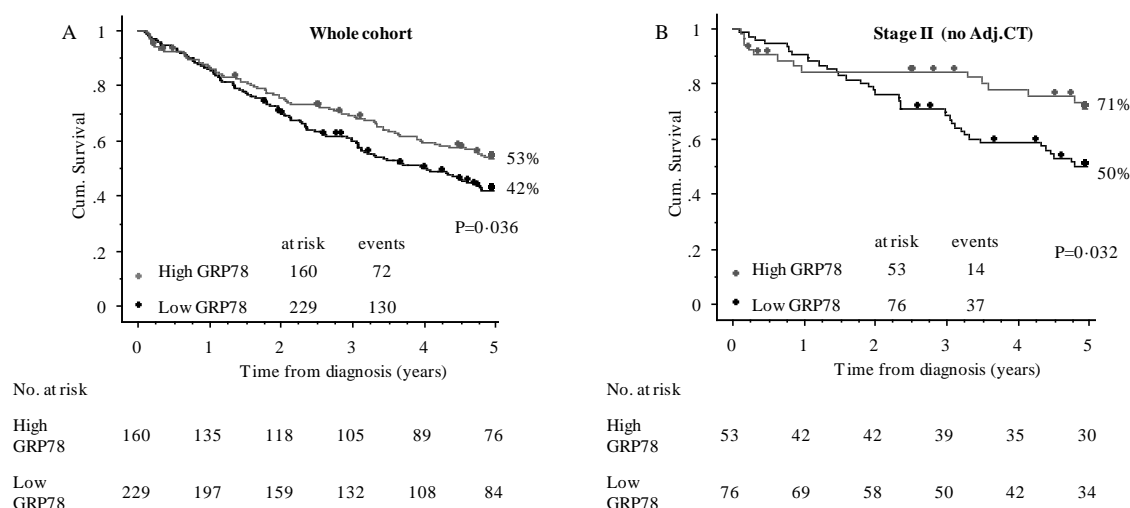


Figure 3.1.8-1: Kaplan-Meier plots showing overall 5yr survival according to GRP78 expression in the whole cohort (A); Stage II cancers (excluding patients who received adjuvant chemotherapy) (B). (Adj.CT=adjuvant chemotherapy)

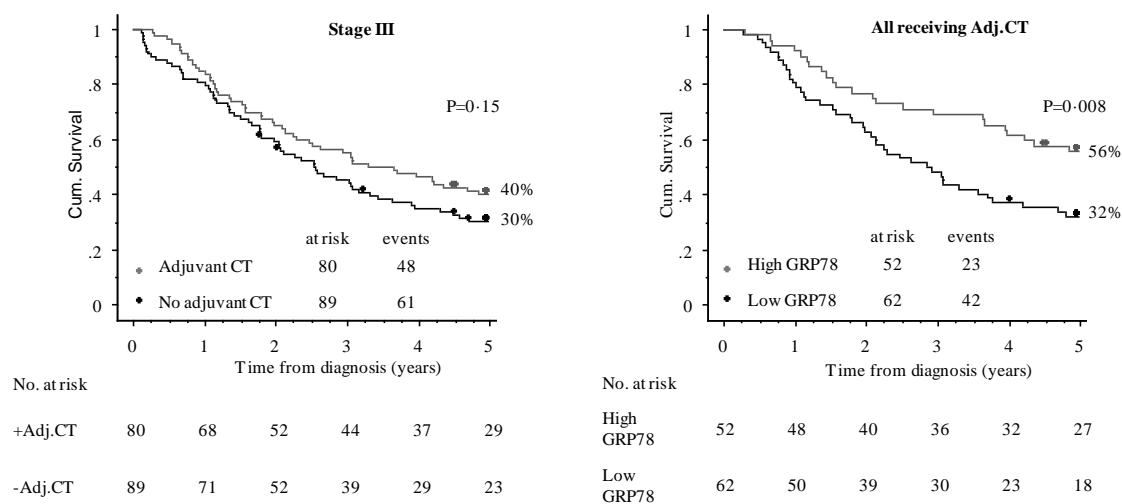


Figure 3.1.8-2: Kaplan-Meier plot demonstrating, (A) overall survival according to treatment in Stage III cancers; (B) overall survival in those who received adjuvant chemotherapy (all stages) according to GRP78 expression.

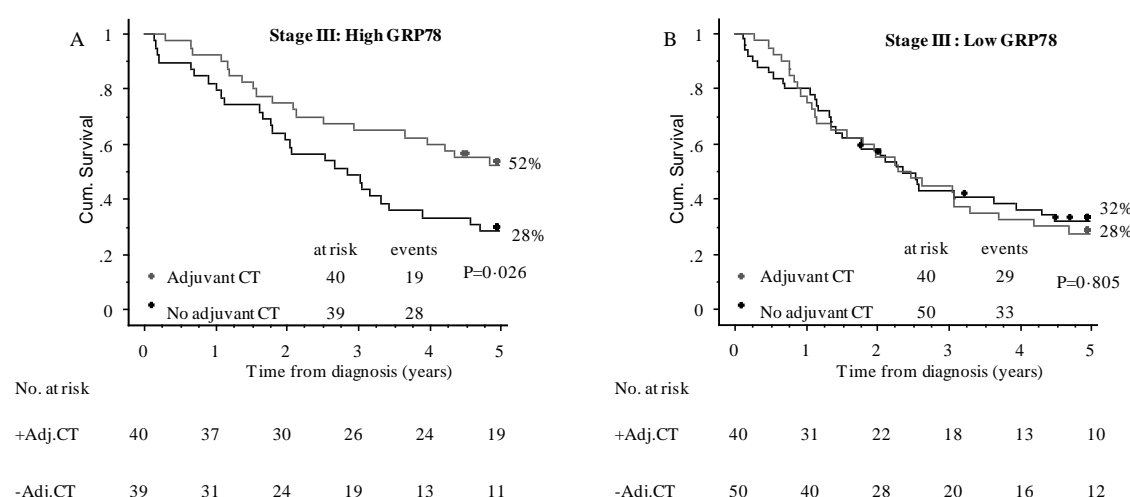


Figure 3.1.8-3: Kaplan-Meier plots showing overall survival according to treatment in Stage III cancers with high GRP78 (A), or low GRP78 (B).

(Adj.CT=adjuvant chemotherapy)

3.1.9 Univariate and Multivariate analysis

Following the observation that patients whose tumours exhibited elevated GRP78 expression had better overall 5yr survival than those with low GRP78, a univariate and multivariate Cox proportional hazards regression analysis was performed to see if GRP78 was an independent prognostic indicator in this cohort. The Cox model was constructed as described in section 2.8 on page 97.

Multivariate analysis demonstrated low GRP78 to be an independent prognostic indicator of overall 5-year survival ($P=0.004$; $HR=1.551$; 95%CI 1.155-2.082). Age ($P=0.009$; $HR=1.017$; 95%CI 1.004-1.030), positive nodal status ($P<0.001$; $HR=1.871$; 95%CI 1.399-2.502), complete excision ($P=0.01$; $HR=0.585$; 95%CI 0.390-0.878) and poor differentiation ($P=0.035$; $HR=1.703$; 95%CI 1.039-2.791) were also found to be independent prognostic indicators (Table 3.1-6).

As a key finding of the survival analysis was the observation that patients who benefitted the most from adjuvant chemotherapy were those with elevated GRP78, the univariate and multivariate analysis were repeated on the subgroup of 114 patients who received chemotherapy following surgery. The results are demonstrated below in Table 3.1-7. Weak GRP78 expression remains a significant predictor of poor prognosis in the subgroup of patients who received adjuvant chemotherapy ($P=0.016$; $HR=1.896$; 95%CI 1.128-3.189). This observation suggests GRP78 has value as a treatment predictive biomarker.

In contrast, when a similar analysis is performed on patients treated by surgery alone (all stages), with no adjuvant chemotherapy, (Table 3.1.9-) the prognostic value of GRP78 was not as robust in the Cox analysis ($P=0.067$; $HR=1.393$; 95%CI 0.977-1.988). This raises doubt as to the value of GRP78 as a potential prognostic biomarker when applied across all stages of disease. However, when this analysis was restricted to those with Stage II disease, weak GRP78 did predict poorer prognosis ($P=0.015$; $HR=2.164$; 95%CI 1.165-4.021).

Table 3.1-6: Association between GRP78 expression and overall 5 year survival for the whole cohort.

Variable	n	Univariate				Multivariate			
		HR	95%CI	χ^2	P-value	HR	95%CI	χ^2	P-value
Age	389	1.012	0.999-1.025	3.157	0.076	1.017	1.004-1.030	6.856	0.009
Tumour size									
<60mm	271	0.893	0.662-1.206	0.542	0.462	-	-	-	-
>60mm	118	1	-	-	-	-	-	-	-
pT									
T1	19	0.452	0.187-1.091	3.122	0.077	-	-	-	-
T2	58	0.934	0.564-1.545	0.072	0.789	-	-	-	-
T3	262	0.836	0.557-1.257	0.739	0.39	-	-	-	-
T4	50	1	-	-	-	-	-	-	-
pN									
N0	220	1	-	-	-	1	-	-	-
N1-N2	169	1.93	1.462-2.547	21.549	<0.001	1.871	1.399-2.502	17.828	<0.001
Resection margin									
Clear	345	0.538	0.365-0.794	9.759	0.002	0.585	0.390-0.878	6.720	0.01
Involved	43	1	-	-	-	1	-	-	-
Differentiation									
Well/Mod	358	1	-	-	-	1	-	-	-
Poor	27	1.717	1.070-2.756	5.023	0.025	1.703	1.039-2.791	4.458	0.035
Grp78 expression									
Weak	229	1.359	1.019-1.813	4.356	0.037	1.551	1.155-2.082	8.525	0.004
Elevated	160	1	-	-	-	1	-	-	-

HR, Hazard Ratio; CI, confidence interval

Table 3.1-7: Association between GRP78 expression and overall 5 year survival for patients who received adjuvant chemotherapy.

Variable	n	Univariate				Multivariate			
		HR	95%CI	χ^2	P-value	HR	95%CI	χ^2	P-value
Age	114	1.027	0.996-1.058	2.931	0.087	1.03	0.998-1.063	3.329	0.068
Tumour size									
<60mm	78	1.238	0.719-2.133	0.593	0.441	-	-	-	-
>60mm	36	1	-	-	-	-	-	-	-
pT									
T1	2	0.924	0.121-7.066	0.006	0.939	-	-	-	-
T2	10	1.424	0.567-3.576	0.565	0.452	-	-	-	-
T3	81	0.898	0.484-1.668	0.116	0.734	-	-	-	-
T4	21	1	-	-	-	-	-	-	-
pN									
N0	34	1	-	-	-	-	-	-	-
N1-N2	80	1.372	0.789-2.386	1.255	0.263	-	-	-	-
Resection margin									
Clear	93	1.338	0.681-2.627	0.714	0.398	-	-	-	-
Involved	20	1	-	-	-	-	-	-	-
Differentiation									
Well/Mod	107	1	-	-	-	1	-	-	-
Poor	5	3.240	1.294-8.112	6.300	0.012	2.927	1.145-7.481	5.032	0.025
Grp78 expression									
Weak	62	1.964	1.179-3.271	6.716	0.01	1.896	1.128-3.189	5.825	0.016
Elevated	52	1	-	-	-	1	-	-	-

HR, Hazard Ratio; CI, confidence interval

Table 3.1.9-3: Association between GRP78 expression and overall 5 year survival for patients treated by surgery alone.

Variable	n	Univariate				Multivariate			
		HR	95%CI	χ^2	P-value	HR	95%CI	χ^2	P-value
Age	275	1.015	0.998-1.032	3.086	0.079	1.01	0.993-1.026	1.334	0.248
Tumour size									
<60mm	193	0.773	0.538-1.109	1.961	0.161	-	-	-	-
>60mm	82	1	-	-	-	-	-	-	-
pT									
T1	17	0.414	0.150-1.140	2.915	0.088	-	-	-	-
T2	48	0.857	0.454-1.618	0.226	0.634	-	-	-	-
T3	181	0.819	0.474-1.415	0.512	0.474	-	-	-	-
T4	29	1	-	-	-	-	-	-	-
pN									
N0	186	1	-	-	-	1	-	-	-
N1-N2	89	2.310	1.646-3.243	23.419	<0.001	2.099	1.480-2.977	17.311	<0.001
Resection margin									
Clear	252	0.250	0.154-0.407	31.215	<0.001	0.281	0.170-0.465	24.572	<0.001
Involved	23	1	-	-	-	1	-	-	-
Differentiation									
Well/Mod	251	1	-	-	-	-	-	-	-
Poor	22	1.522	0.875-2.647	2.215	0.137	-	-	-	-
Grp78 expression									
Weak	167	1.172	0.826-1.662	0.791	0.374	1.393	0.977-1.988	3.351	0.067
Elevated	108	1	-	-	-	1	-	-	-

HR, Hazard Ratio; CI, confidence interval

3.1.10 siRNA knockdown of GRP78

No other studies to date have examined the consequences of GRP78 expression and sensitivity to 5-FU in colon cancer cells. The observation of an association between low GRP78 and worse outcome following treatment with fluorouracil-based adjuvant chemotherapy in the Liverpool cohort suggests a possible functional relationship between GRP78 and response to 5-FU. This is potentially highly relevant as there are no biomarkers presently available that can predict response to 5-FU, and furthermore, the potential applicability of a cheap and simple immunohistochemical test makes GRP78 a very attractive potential biomarker.

I performed in-vitro experiments using siRNA-transfection to elucidate a potential mechanistic relationship between GRP78 and 5-FU sensitivity. From the observation in our cohort, I hypothesised that a depletion of endogenous GRP78 would reduce the efficacy of 5-FU in vitro.

I selected the HCT116p53wt cell line for several reasons. They have documented sensitivity to 5-FU in vitro, are easy to transfect with siRNA and in preliminary experiments, exhibited elevated levels of GRP78 in a non-induced state and gave good results on the flow cytometer as they are easy to harvest by trypsinisation.

A set of 4 siGENOME siRNA targeting GRP78 with documented efficacy were purchased from Dharmacon. HCT116p53wt cells were transfected and the transfection efficiency was assessed by western blot to see which siRNA reagent produced the most effective GRP78 knockdown. I was interested to see if GRP78 depletion remained apparent at 72 hours post transfection as this was the time point at which cells were to be harvested for further experiments. Short interfering RNA (siRNA) oligonucleotide knock-down experiments were performed as described in

section 2.4.1 on page 80, using a final siRNA concentration of 40 nM. A mock transfection control was prepared identically except for omitting the siRNA oligonucleotide.

GRP78 levels were depleted by all four of the siRNA reagents selected, and this remained apparent after 72 hours (Figure 3.1.10-1), but siGENOME D-008198-03 produced the most effective knockdown and was selected for further use.

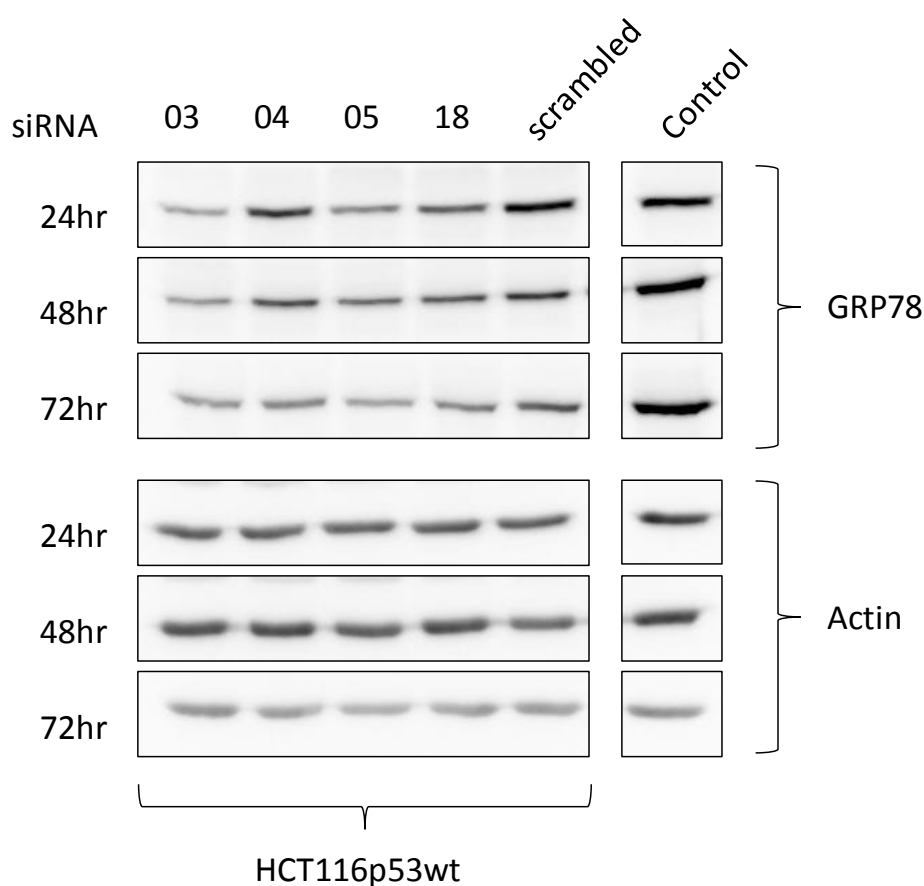


Figure 3.1.10-1: Western blot demonstrating GRP78 depletion after transfection with a panel of GRP78-targeting siRNA in HCT116p53wt cells.

Cells were transfected with 40nM scrambled siRNA or GRP78 siRNA using Lipofectamine 2000 as a transfection reagent. Mock transfected cells were treated identically except for omission of the siRNA oligomer. Cells were harvested at 24, 48 and 72 hrs and cell lysates subjected to western blot. Transfection efficiency was detected using a rabbit polyclonal antibody specific for GRP78 (sc13968, Santa Cruz). Actin was used as a loading control. (03 = siGENOME D-008198-03; 04 = siGENOME D-008198-04; 05 = siGENOME D-008198-05; 18 = siGENOME D-008198-18; all from Dharmacon).

3.1.11 siRNA knockdown of GRP78 reduces cell apoptosis in response to 5-FU

I examined the effect of depleting endogenous GRP78 using siRNA in HCT116 cells. Two days following mock treatment or transfection with GRP78-targeting or scrambled siRNAs, cells were treated +/-100 μ M 5-FU (the concentration required to cause 50% growth inhibition of HCT116 cells at 24 hours).(341)

When HCT116 cells were transfected with GRP78 siRNA (siGENOME D-008198-03) or a scrambled siRNA control, a 10% increase in cell apoptosis was measured as compared with a mock treated control. However, cells transfected with GRP78 siRNA demonstrated substantially less 5-FU-induced apoptosis than the scrambled siRNA transfected or mock treated control at 24hrs, 19.81% versus 52.4% and 48.54% respectively (Figure 3.1.11-1), as demonstrated by Annexin V apoptosis assay (section 2.7.4.). It is notable that the level of apoptosis in 5-FU treated cells with reduced GRP78 expression is comparable to that observed in cells in the absence of 5-FU treatment (Figure 3.1.11-1B), suggesting an important role for GRP78 in 5-FU induced apoptosis in these cells. Thus it appears that GRP78 expression levels determine cellular sensitivity to 5-FU treatment and thus my in vitro data accord with the clinical data and suggest a deterministic role for GRP78 in the adjuvant response. This experiment was repeated using a different siRNA targeting GRP78 (siGENOME D-008198-05), with similar findings (see Appendix section 6.3).

Analysis of knockdown efficiency was performed at the protein level by standard SDS-PAGE and western blotting for each experiment (Figure 3.1.11-2A).

In support of the above findings, decreased cleaved PARP levels were detected in 5-FU-treated GRP78 siRNA-transfected HCT116p53wt cells at 24 hours relative to 5-

FU-treated mock treated or scrambled siRNA controls (Figure 3.1.11-2B). Together, these results indicate that GRP78 expression promotes apoptosis in response to 5-FU.

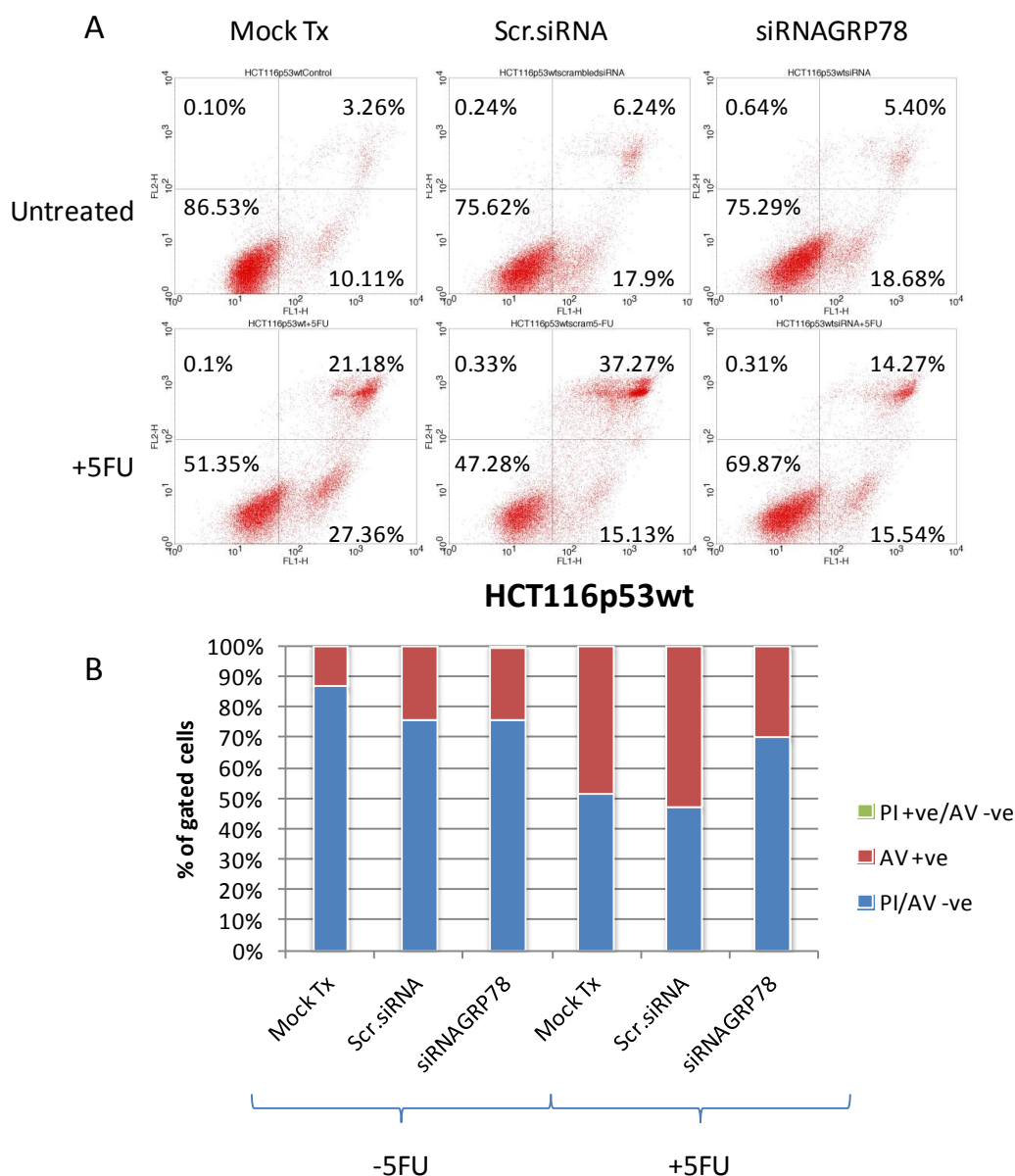


Figure 3.1.11-1: siRNA knockdown of GRP78 attenuates the apoptotic response to 5-FU.

Cells were seeded 24 hours prior to transfection with a final siRNA concentration of 40 nM. A mock transfection control was prepared identically except for omitting the siRNA oligonucleotide. 48 hr after transfection, cells were treated +/- 100 μ M 5-FU (medac GmbH, Germany) for 24 hr then harvested for flow cytometry. (A) Bivariate distributions of Annexin V (AV)-positivity (apoptosis, FL1 parameter, x-axis) vs. Propidium iodide (PI)-positivity (necrosis, FL2 parameter, y-axis) were generated for each population. Viable cells were measured as those in the lower left quadrant (PI/AV -ve); early apoptotic cells are in the right lower quadrant (PI -ve/AV +ve, late apoptotic cells are in the right upper quadrant (PI +ve/AV +ve); remaining cells in the left upper quadrant were deemed necrotic or dead (PI +ve/AV -ve). (B) These analyses were used to generate percentages of viable (PI/AV -ve), apoptotic (AV +ve) or dead (PI +ve/AV -ve) cells at 24 hr of treatment.

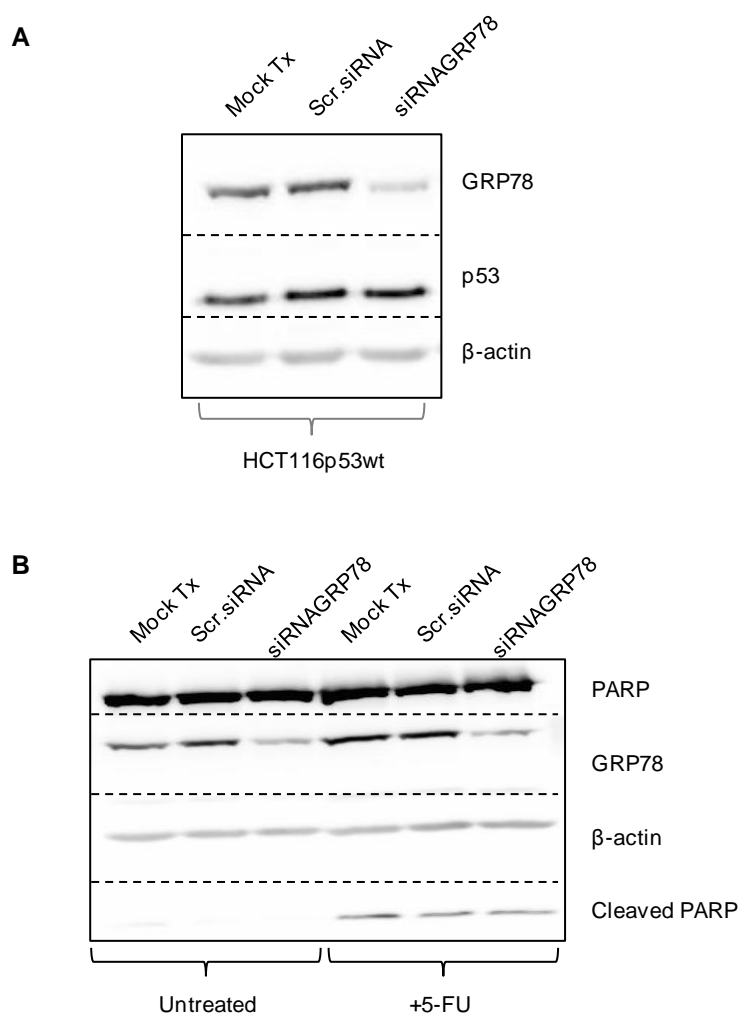


Figure 3.1.11-2: siRNA knockdown of GRP78 results in reduced PARP cleavage following exposure to 5-FU.

(A) Western blot confirming GRP78 knockdown by siRNA transfection compared with mock treated and scrambled siRNA controls, in HCT116 p53 wild-type cells. (B) Mouse monoclonal anti-PARP antibody that recognises both the cleaved and uncleaved isoform (BD Transduction) reveals PARP cleavage for drug-treated HCT116p53wt cells at 24 hours of treatment with 100 μ M 5-FU. Actin was used as a loading control.

3.1.12 Cell cycle analysis following siRNA knockdown of GRP78 and treatment with 5-FU.

Following GRP78 depletion by siRNA, cell cycle analysis of treated or untreated HCT116 cells was performed (Figure 3.1.12-1) using propidium iodide as detailed in section 2.7.2 on page 91.

In the p53 wild-type setting, treatment of HCT116 cells with 100 μ M 5-FU for 24 hours resulted in an increase in the number of cells in S-phase, together with a reduction of G1 and G2/M phase cells. This response to 5-FU is consistent with previous reports.(342-344) An accumulation of cells in sub G1 is also noted following exposure to 5-FU. It is notable that the cell cycle profile was not significantly affected by GRP78 siRNA transfection.

In contrast, p53 null HCT116 cells demonstrated no such S-phase arrest after exposure to 5-FU (Figure 3.1.12-2), but did show a reduction of cells in G2/M, largely as a result of an increased number of cells in G1. This G1 arrest may contribute to resistance to 5-FU induced apoptosis in these p53 null cells.(126) There were far fewer cells in sub G1 in p53 null cells treated with 5-FU relative to drug treated p53 wild-type cells. Again, as in the wild-type p53 cells, GRP78 knockdown did not significantly affect the cell cycle.

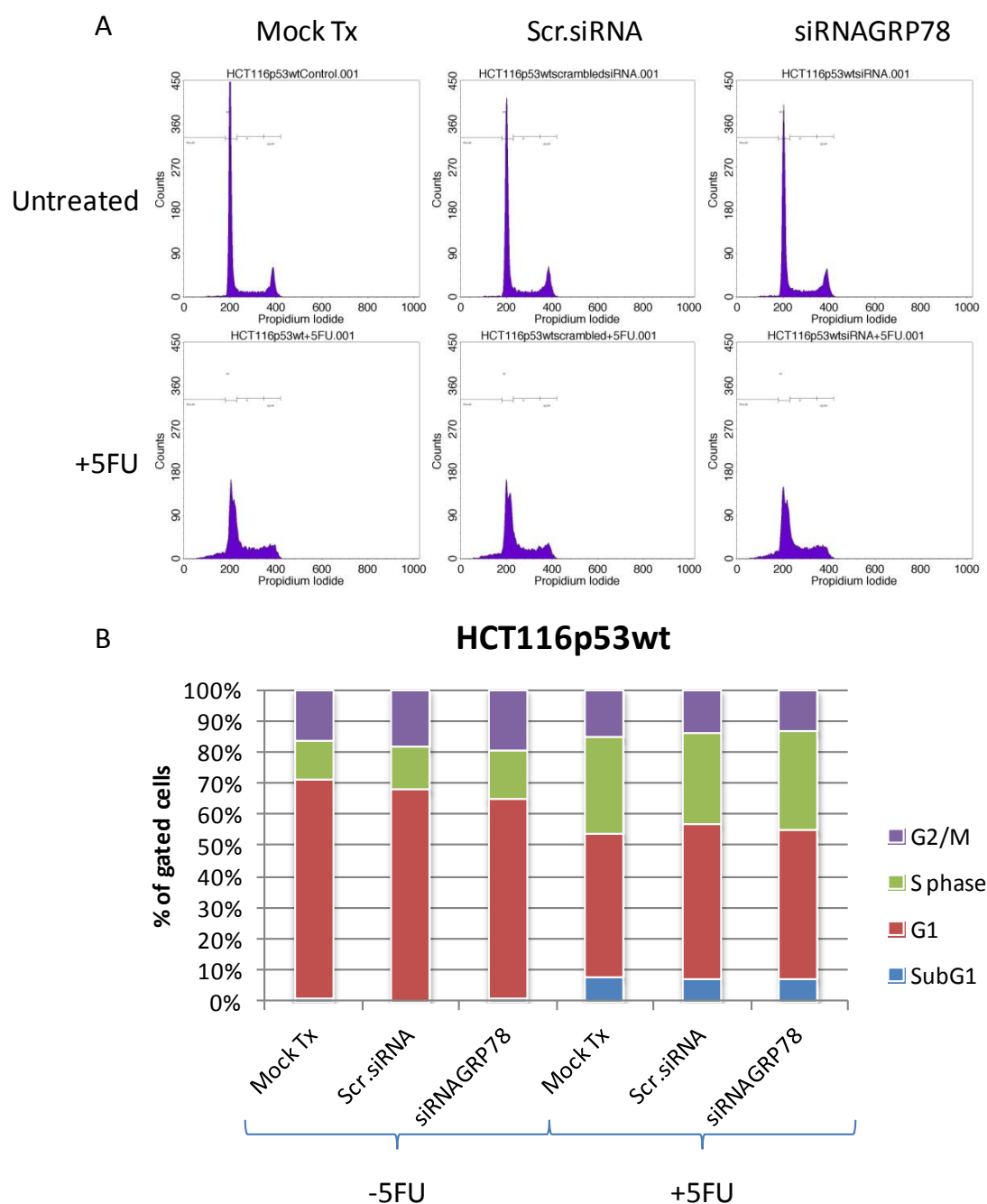


Figure 3.1.12-1: siRNA knockdown of GRP78 has minimal effect upon the cell cycle.

(A) DNA content was measured on asynchronously growing cells using propidium iodide staining 72 hr following transfection with siRNA GRP78 or mock/scrambled controls. Cells were exposed to 100 μ M 5-FU or vehicle control for 24 hr prior to harvesting. (B) The percentages of cells in various stages of the cell cycle. Sub G1 indicates dead cells with fragmented DNA, G1 indicates cells in the quiescent state of the cell cycle, S phase is a period of DNA synthesis, and G2/M indicates the period of rapid growth and mitosis.

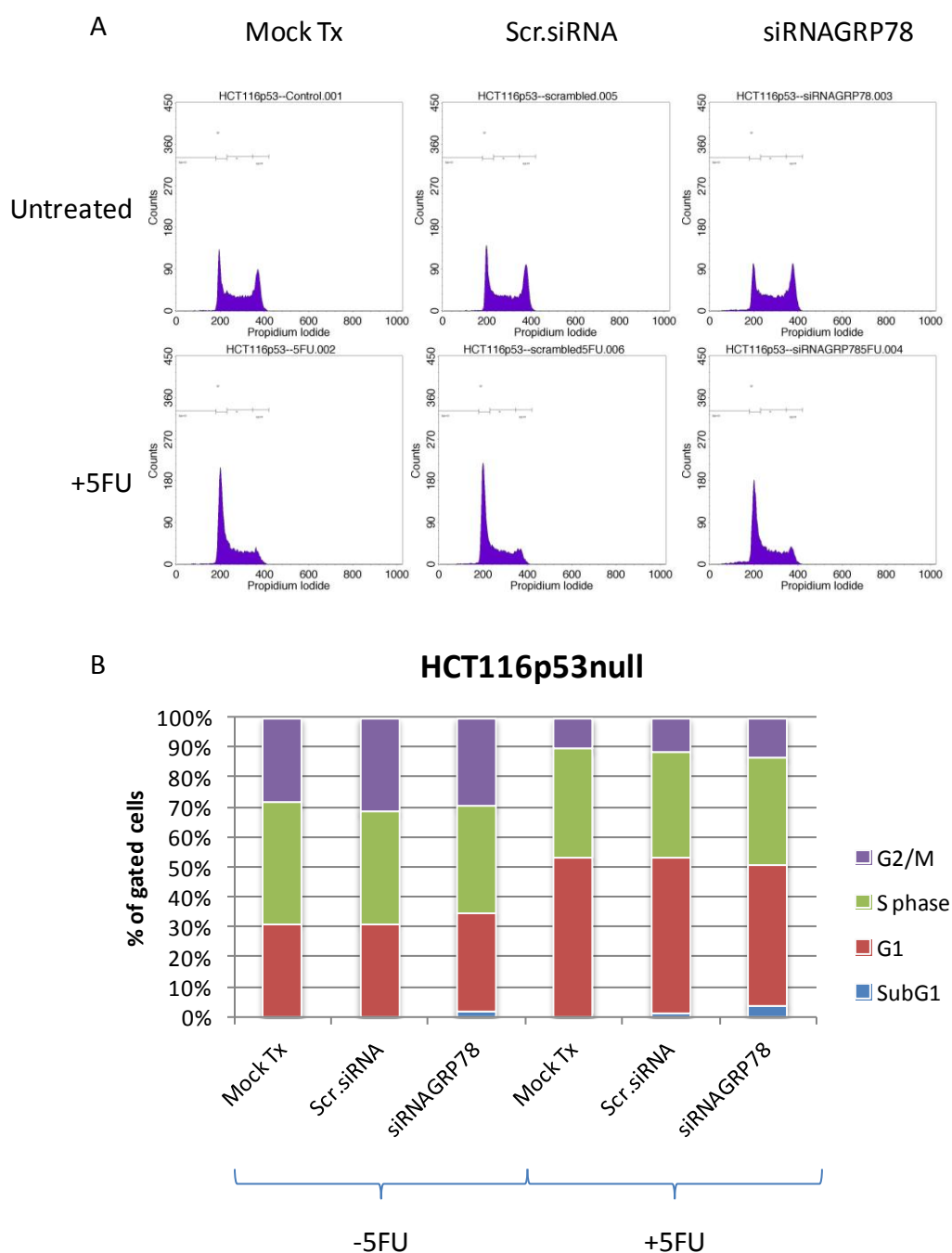


Figure 3.1.12-2: siRNA knockdown of GRP78 has minimal effect upon the cell cycle in p53 null HCT116 cells.

(A) Cell cycle profile of HCT116p53null cells treated in the same manner as in the legend for Figure 3.1.12-1. DNA content was measured on asynchronously growing cells using propidium iodide staining 72 hr following transfection with siRNA GRP78 or mock/scrambled controls. Cells were exposed to 100 μ M 5-FU or vehicle control for 24 hr prior to harvesting. (B) The percentages of cells in various stages of the cell cycle.

3.2 Discussion 1

This study represents the largest series of colorectal cancers examined for GRP78 expression and the first to examine its relationship to survival and its potential as a predictive biomarker for adjuvant chemotherapy. Having established a role for GRP78 in sensitivity to 5-FU in-vitro, we present evidence that GRP78 is elevated in colorectal cancer, is an independent prognostic indicator, and a positive predictor of benefit from fluoropyrimidine-based adjuvant chemotherapy.

The reported 5-year survival rate for stage II disease is 75% after surgery but a subgroup of patients have survival similar to stage III patients of 50%.⁽³⁴⁵⁾ The data presented here are in keeping with this with low GRP78 stage II patients showing overall 5 year survival of 50% (compared with 71% with high GRP78). Contemporary clinicopathological high risk features for stage II disease such as elevated carcinoembryonic antigen, obstruction or perforation, T4 stage, inadequate nodal resection (<12 nodes), lymphovascular invasion, and high-grade disease may predict worse outcome, but they do not predict response to chemotherapy.⁽⁸⁴⁻⁸⁶⁾ Importantly, although low GRP78 can predict a poor prognostic group, our data from stage III disease suggest these patients may not benefit from a fluoropyrimidine-based regimen.

Following recent negative reports,^(96-98, 346) the role of new targeted therapies for adjuvant treatment remains uncertain and thus, there continues to be reliance upon fluoropyrimidine-based chemotherapy as a mainstay of drug treatment for colorectal cancer. Patients with stage III disease and elevated GRP78 had a 20% higher 5-year survival compared with patients with low GRP78 who received the same adjuvant

treatment. Indeed, in this cohort, patients with low GRP78 failed to benefit from adjuvant chemotherapy. This important finding warrants further investigation. The possibility of predicting a treatment group with a higher risk of treatment failure, such as those with tumours expressing low levels of GRP78, could allow better selection and counselling of patients for targeted combination therapy, although this would require validation in prospective clinical trials.

Another group recently reported that GRP78 can serve as a predictive biomarker for response to doxorubicin/taxane-based adjuvant chemotherapy in breast cancer.(347) When considered with our data, this may necessitate a re-evaluation of our understanding of the role of GRP78 in cancer. With a few exceptions,(285, 286) GRP78 expression in human cancers is typically associated with more aggressive phenotype and poor prognosis.(275-277, 280, 287, 296, 333, 334) The influence of GRP78 is likely to be tissue and drug specific, however, some studies suffer from small numbers of enrolled patients, heterogeneous tissue preparation, use of different GRP78 antibodies (in some cases not specific for GRP78 as we have demonstrated), and different scoring systems. Furthermore, in many cases, analysis did not extend to patients who had received adjuvant therapy. Similarly, in-vitro studies typically report that ER-stress leads to anti-apoptotic properties and drug resistance.(276, 296) However, the difficulty of in-vitro modelling of GRP78 expression is that, in many cases, the conditions used to induce ER-stress, such as glucose deprivation or pharmacological influence, result in cell cycle arrest,(348) a well recognised cause of drug resistance,(349) and therefore make interpretation of simple assays that only measure proliferation problematic (see Appendix 6.4 Difficulties of modulating GRP78 and assessing chemosensitivity in-vitro). These conditions almost certainly

fail to accurately model the prolonged physiological stress encountered during tumour growth. Methods not reliant upon cell proliferation, such as flow cytometry, may be superior for detecting alteration in chemosensitivity upon manipulation of GRP78. We chose to perform siRNA knockdown of GRP78 that had minimal effect on the cell cycle, rather than inducing ER-stress which might otherwise confound interpretation through the pleiotropic effects of the stress. As these experiments are based upon endogenous levels of GRP78, therefore we are modulating physiologically relevant levels of GRP78.

Much remains to be elucidated regarding how UPR activation elicits such different cellular responses. It is possible that in patients with high GRP78, tumours are primed toward pro-apoptotic pathways of the UPR and, as such, are more susceptible to chemotherapy. Alternatively, one can envisage a model in which cells that have successfully adapted to the tumour microenvironment demonstrate persistently elevated expression of GRP78 and are therefore better equipped to process or remove proteins from the ER, thus preventing accumulation of misfolded proteins and avoiding activation of the UPR and cell-cycle arrest. This allows continued proliferation and thus sensitivity to cytotoxic agents. In contrast, the ER has a reduced capacity to handle protein in cells with low levels of GRP78, so although cellular function can be maintained in the absence of increased protein burden, these cells are more sensitive to perturbations in the tumour microenvironment that trigger the prosurvival arm of the UPR. Whilst *in vitro* this can lead to inhibition of translation and cell cycle arrest, the *in vivo* consequences of this are not yet understood and may well lead to altered tumorigenicity. In this scenario, cells with low GRP78 are therefore more likely to display resistance to 5-FU. Models

proposing adaptation to chronic ER stress such as this are not novel,(350) and furthermore, it has been demonstrated that over-expression of GRP78 in vitro is tolerated and, for example, does not result in cell cycle alteration.(289) The observation that tumours expressing low GRP78 did not seem to respond to treatment with 5-FU suggests that these cells are concentrating resources on mitigating stress rather than on growth and division, in keeping with the siRNA knockdown experiment presented here. We speculate that tumours with elevated GRP78 may have successfully adapted to their microenvironment through induction of chaperones and modulation of their metabolic state,(229) thus allowing continued proliferation and sensitivity to cytotoxic agents. In the case of tumours with low availability of GRP78, when further stress is applied, the protein-folding capacity of the ER becomes overwhelmed and the UPR is activated leading to cell cycle arrest and drug resistance. However, our results raise the question of whether GRP78 may contribute to chemosensitivity through mechanisms other than direct activation of the UPR. In support of this, recent developments suggest that GRP78, traditionally believed to be an ER-resident, can be identified in the cytoplasm and on the cell surface of cancer cells, and furthermore, is biologically active and may affect cell viability at both of these sites.(351) We must also be cautious that the observations made in vitro, using specialised cell lines, may not always reflect the true in vivo endogenous mechanisms.

In view of the multiple genetic alterations which define the evolution of colorectal cancer,(24) much attention is currently focused on identifying molecular or genetic factors that may help predict survival or response to chemotherapy. Mutation of p53, KRAS mutation, defective DNA mismatch repair (dMMR) and loss of

heterozygosity on the long arm of chromosome 18 are all important steps in the development of colorectal cancers.(352) However, examination for genetic mutations such as these is often complex and expensive and in view of many conflicting reports in the literature, with the exception of KRAS,(353) the routine use of these biomarkers is not currently recommended.(87) A recent study using tissue from the QUASAR study has also failed to identify biomarkers predictive of benefit from chemotherapy.(353) GRP78 is easily detectable by immunohistochemistry using highly specific commercially available antibodies and may provide a simple and cheap alternative tool for risk stratification.

Clearly, the gold standard for evaluating a potential predictive biomarker such as GRP78 is a prospective randomised-controlled trial. Nonetheless, our study has several important strengths. Firstly, the prospective uniform nature of specimen collection for all subjects by our pathology department minimises potential systematic bias from this study. The study cohort was essentially a random sample of the expected target population and the chemotherapy delivered represents real world practice. Secondly, we used an antibody that we were able to validate using siRNA and EGFSubA whereas some antibodies to GRP78 cross react with related, potentially more abundant, proteins such as HSP72 as shown. Finally, our clinical findings support our *in vitro* data demonstrating a functional role for endogenous and thus physiologically relevant levels of GRP78 in cellular responses to 5-FU.

In this study, overall survival (OS) was chosen as an unambiguous and clinically relevant end point. Traditionally in clinical trials, OS rate at 5 years has been the most typically quoted metric for judging the success or a particular treatment regimen. It is easy to measure and interpret, and clinically meaningful. A major

drawback of this approach however is that in a retrospective non randomised, cohort study such as this, it is not entirely possible to isolate any causal effect of the GRP78 on the efficacy of fluorouracil-based therapy from the many other factors that may have influenced treatment decisions at the time, for example, age, comorbidity, patient choice, etc. In this Liverpool cohort, analysis reveals that those receiving adjuvant chemotherapy were on average 8.75 years younger than those who did not (61.71yr vs. 70.46yrs, $p < 0.001$). Thus, other factors may be having an influence on overall survival. In larger randomised trials, factors such as age, performance status are controlled for by the process of randomisation but this cannot be the case in a retrospective study such as this. Ideally, such retrospective biomarker analysis would be conducted against data from a well-conducted randomised controlled trial (RCT), with samples available from a large number of patients.(354) By example, KRAS was identified as a predictive biomarker in advanced colorectal cancer using validation from a previous RCT.(355) Even the multivariate analysis does not completely account for these confounding effects on survival. Progression-free survival (PFS) is increasingly used in RCT's of new agents in cancer treatment and in the case of colorectal cancer, has been proven to be a useful surrogate for overall survival.(356) In a study on adjuvant chemotherapy, it might be argued that the greatest effect will be on PFS as a marker of change in tumour burden in response to treatment. This does not always necessarily convert to a benefit to patients as significant absolute degrees of improvement in PFS may not always translate into meaningful improvement in OS. PFS is more difficult to measure than OS in which the date of death is exact and defining PFS will be biased by timing of follow up measurement and other forms of bias.(357) The retrospective nature of this study

and limitations of access to accurate mortality data meant that overall survival was the most viable and robust end point.

A number of questions need to be addressed. Can patients with stage II disease and high GRP78 be spared adjuvant chemotherapy regardless of clinical or pathological parameters or conversely, might these be the subgroup of stage II patients who do benefit from adjuvant 5-FU? Are patients with low GRP78 likely to benefit from combination or non-fluoropyrimidine-based chemotherapy? These questions need answering through larger prospective studies.

In conclusion, the expression of GRP78 in colorectal cancer is an independent marker of survival in colorectal cancer and may be especially useful in identifying a poor prognostic group in stage II disease. In addition, GRP78 predicts and determines response to fluoropyrimidine-based adjuvant chemotherapy allowing identification of a group with a high chance of failure to respond who could be offered alternative therapy.

3.3 Addendum

The external examiner has pointed out that the prognostic and treatment predictive modelling in chapter 3 is incomplete, which has led to over-interpretation of the role of GRP78 expression. The conclusion of chapter 3 should read that the data suggest that GRP78 is a treatment predictive biomarker for mainly 5-FU based adjuvant chemotherapy in patients with colorectal cancer, but GRP78 does not have prognostic characteristics. This interpretation is important to the design of future

studies to further test and quantify the treatment predictive characteristics of GRP78 in patients with colorectal cancer undergoing adjuvant chemotherapy.

4 Pre-clinical investigation of GRP78 as a potential therapeutic target in colon cancer using a novel engineered fusion protein; EGF-SubA.

4.1 Results

4.1.1 Characterisation of Epidermal Growth Factor Receptor (EGFR) expression on a panel of colorectal cell lines.

EGF-SubA has been shown to undergo EGFR-mediated internalisation, in the same manner as EGF, and cytotoxicity to tumour cells via GRP78 cleavage is documented to be dependent upon levels of EGFR expression with PC3 (prostate cancer) or MDA231luc (breast cancer) cells expressing EGFR being substantially more sensitive to this drug than EGFR-negative F98 (engineered rat glioma) or U226-B1 (human myeloma) cells. (314)

To date, the cytotoxicity of EGF-SubA upon colon cancer cells has not been assessed. I therefore sought to characterise EGFR expression in a panel of available colon cancer cell lines prior to investigating the potential cytotoxic effect of EGF-SubA treatment and examine whether this was indeed dependent upon EGFR expression.

As Figure 4.1.1-1 demonstrates, all cell lines express EGFR with the exception of SW620 which is EGFR-deficient. Interestingly, the sister cell line SW480 expresses relatively higher levels of EGFR. This discrepancy is due to a well documented functional knockout of EGFR in SW620 cells, as EGFR copy number is in fact identical to SW480.(358) The p53 status of each cell line was also confirmed in this figure.

As EGF-SubA targets cell surface EGFR, the expression of cell surface EGFR on colon cancer cell lines was assessed using flow cytometry as described in section 2.7.5 (as opposed to western blotting that measures total cellular EGFR).

Flow cytometry revealed that cell surface EGFR expression was present for all cell lines assessed with the exception of SW620 (Figure 4.1.1-2), in keeping with total EGFR as measured by western blot below. This is highlighted in Figure 4.1.1-3.

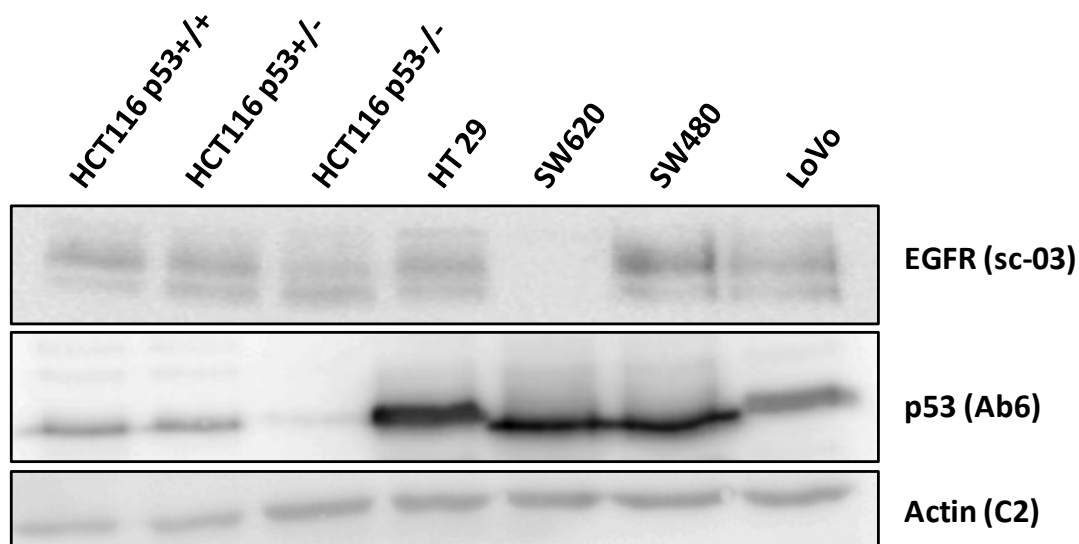


Figure 4.1.1-1: Western blot of EGFR expression in a panel of colon cancer cell lines.

Cells were harvested at 80-90% confluence and cell lysates were subjected to western blot using a rabbit polyclonal antibody for EGFR (Santa Cruz). The p53 status is also examined in this figure using a mouse monoclonal antibody (Oncogene-Merck). Mouse monoclonal antibody for Actin (Santa Cruz) was used as a loading control.

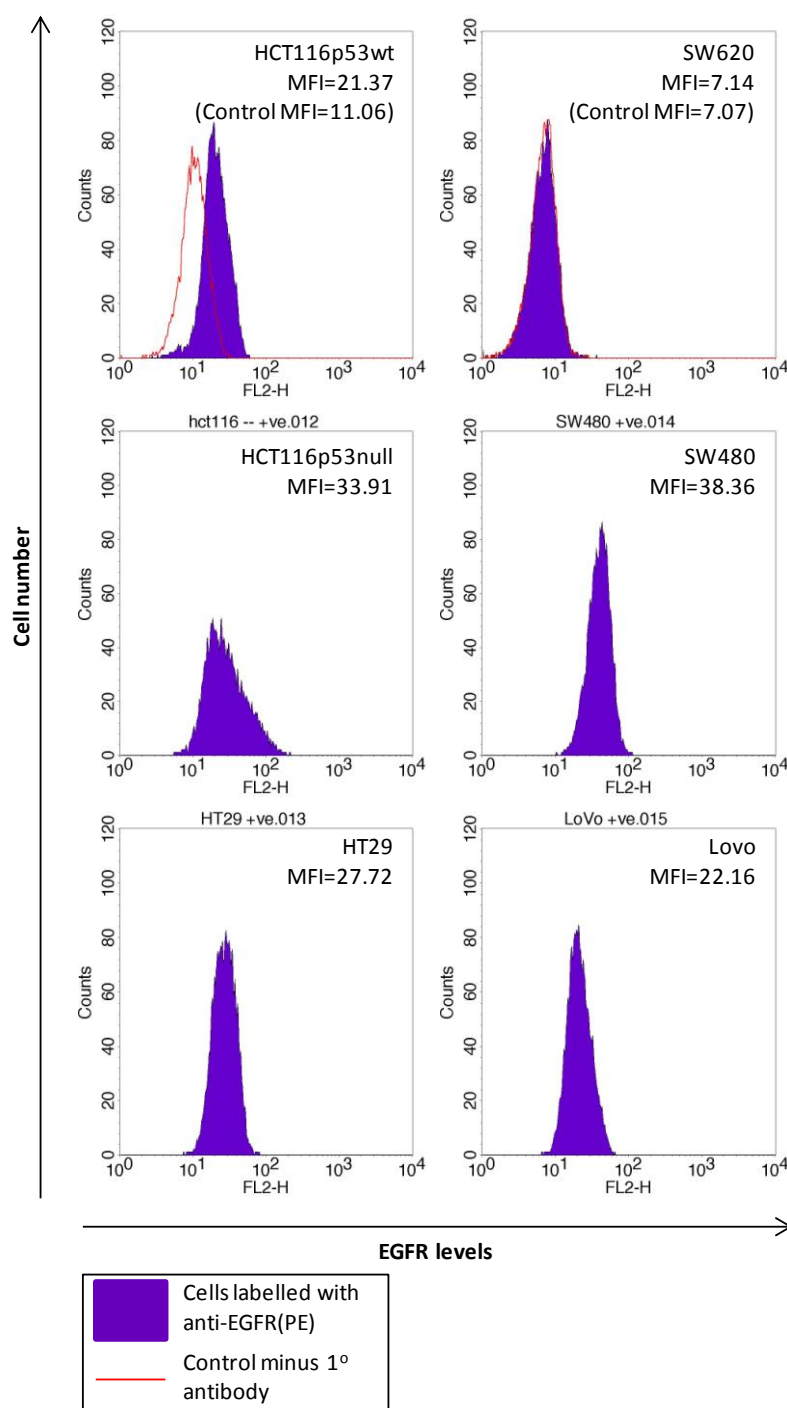


Figure 4.1.1-2: Surface EGFR expression of colon cancer cells as determined by flow cytometry.

Cells were incubated with PE-conjugated anti-human EGFR-specific antibody (abcam) for 30 mins then washed in PBS buffer and analysed by flow cytometry. Mean fluorescent intensity (MFI) is indicated. Controls illustrated for HCT116p53wt and SW620 by way of example were performed by omitting the primary antibody to indicate non-specific fluorescence. EGFR positive HCT116p53wt cells (solid purple fill) show increased MFI versus its control (solid red line), whereas the EGFR negative SW620 show minimal difference to the control.

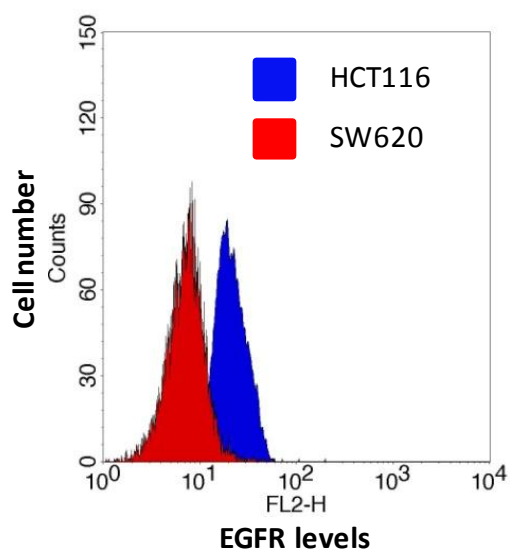


Figure 4.1.1-3: Cell surface EGFR expression of HCT116p53wt (EGFR-positive) and SW620 (EGFR-negative) as determined by flow cytometry.

The histograms have been overlaid for comparison.

4.1.2 EGF-SubA-induced cytotoxicity is EGFR-dependent

In order to assess the inhibitory effects of EGF-SubA upon colorectal cancer cells, I performed a MTT assay as described in section 2.6. Representative growth curves (Figure 4.1.2-1) and dose-response curves (Figure 4.1.2-2) are demonstrated below.

In agreement with the observations of Backer et al. in other cell lines,(314) a negative correlation (Pearson's correlation coefficient = -0.83) was noted between EGFR surface expression and sensitivity to EGF-SubA in colon cancer cell lines (Figure 4.1.2-3). The cytotoxicity of EGF-SubA to colon cancer cells was EGFR-dependent because EGFR-negative SW620 cells were more than 20-fold less sensitive to EGF-SubA than EGFR-positive SW480 cells (Table 4.1-1).

The observed correlation can be quantified using the Spearman rank correlation coefficient which is a non-parametric measure of statistical dependence between two variables.(359) The perfect Spearman correlation of +1 or -1 occurs when each of the variables is a perfect monotone function of the other. If the dependent variable increases as the independent variable increases, the Spearman correlation coefficient is positive; if the dependent variable decreases as the independent variable increases, the Spearman correlation coefficient is negative. Using the data in Table 4.1-1, the Spearman rank correlation coefficient (Rho) = -0.829 ($P=0.064$). However, upon observing the data in Table 4.1-1, HCT116p53null may be regarded as an outlier in that its IC_{50} is comparatively high for its EGFR expression compared with the other cell lines. If HCT116p53null is excluded from the analysis, $Rho=-1.0$ ($P=0.046$), indicating a good correlation.

This raises the question of why the HCT116 p53 null cell line should be an outlier. It is widely accepted that p53 is implicated in numerous processes including cell

growth, differentiation, senescence, apoptosis and angiogenesis.(360) In addition, there is now a body of evidence that p53 has a role in regulating the transcription of the EGFR promoter,(361, 362) and thus it is possible that the p53 null derivative of HCT116, as well as being less sensitive to cytotoxic agents than its p53wt sister, may have altered transcriptional regulation of EGFR.

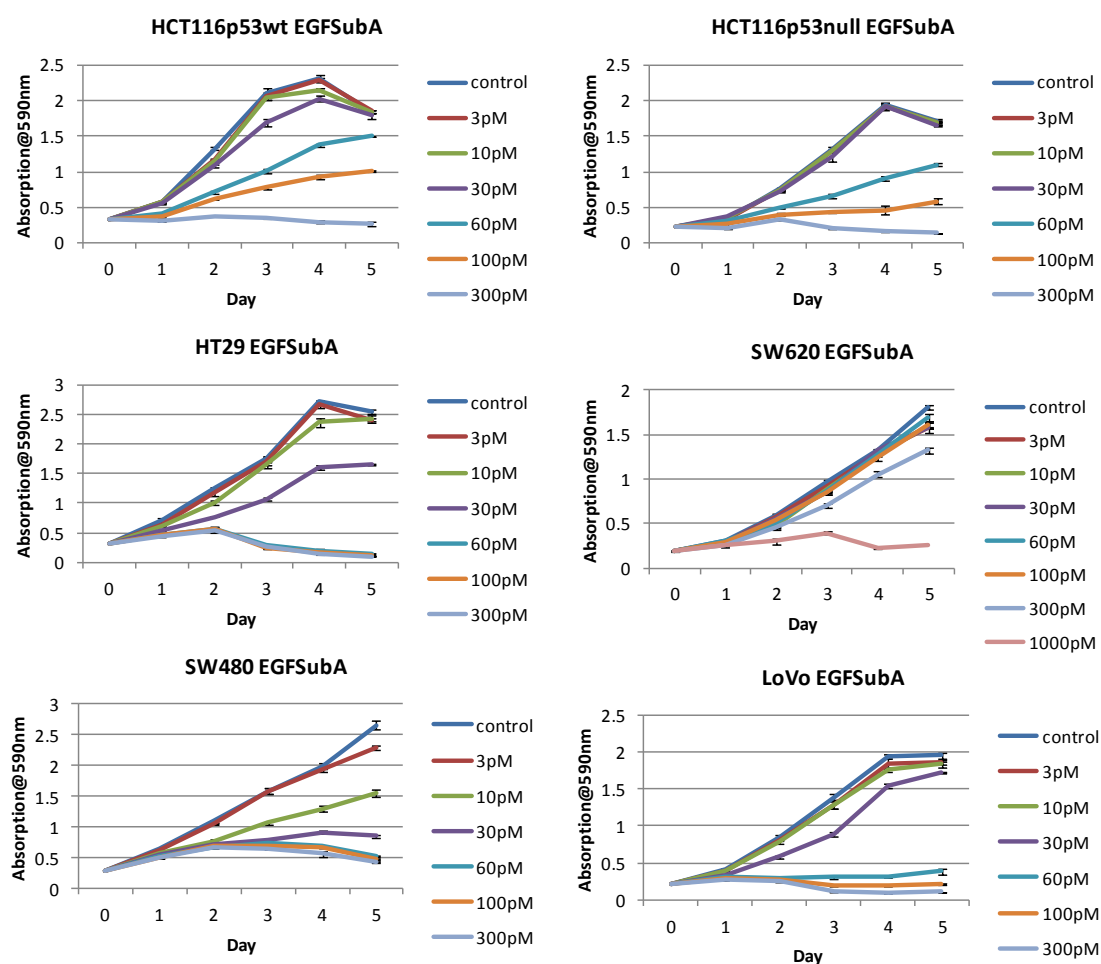


Figure 4.1.2-1: EGF-SubA inhibits growth of colon cancer cell lines at picomolar concentration.

A panel of colon cancer cells, including EGFR positive HCT116, HT29, Lovo and SW480 cells, and the EGFR negative cell line SW620 were seeded onto duplicate 96 well plates at a concentration of 3000 cells/well. After 24 hr, cells were treated with varying concentrations of EGF-SubA or vehicle control (PBS) as illustrated and incubated for 5 days. Media and drug were changed daily. MTT assay was performed daily and scores plotted on a line graph. Error bars represent the standard error from the mean (s.e.m.). for 4 separate wells.

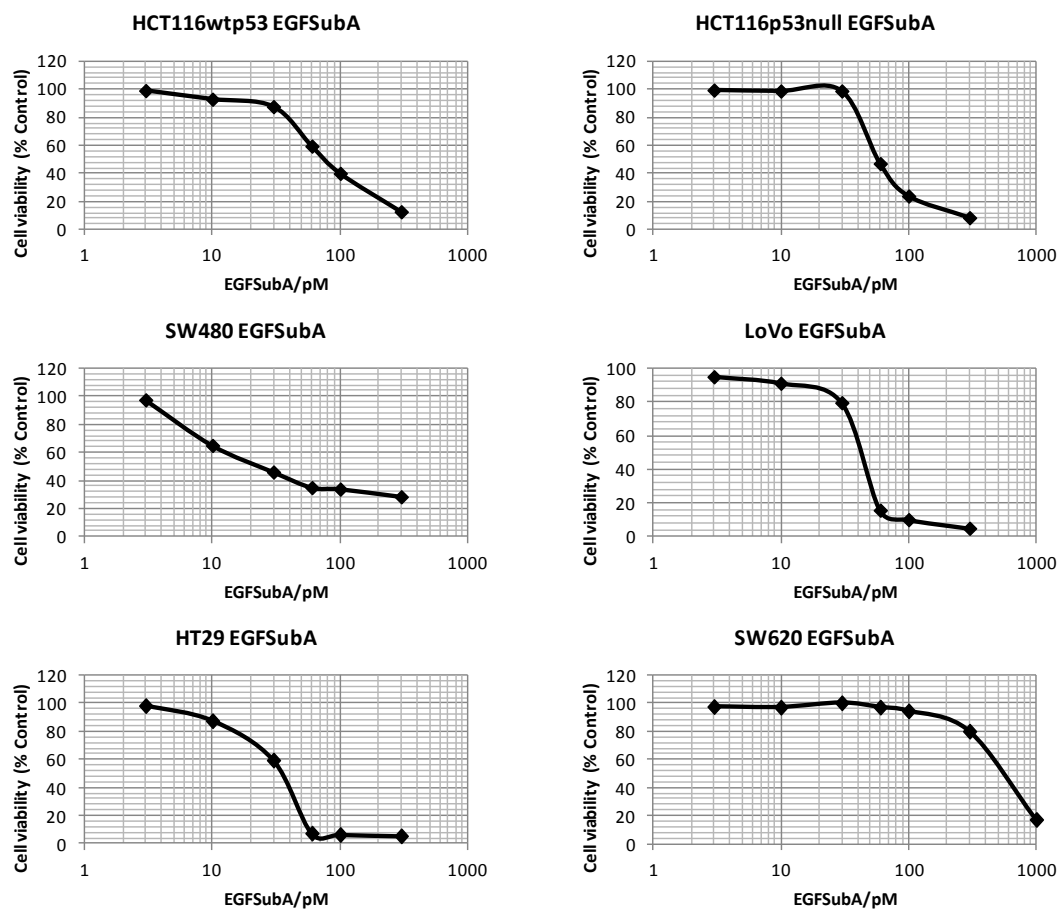


Figure 4.1.2-2: EGF-SubA dose response curves.

Results are extrapolated from growth curves in Figure 4.1.2-1 at day 4.

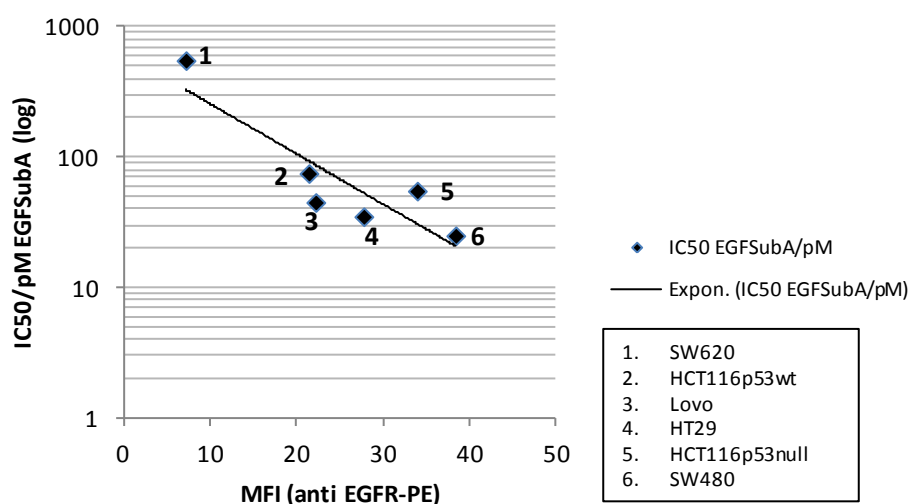


Figure 4.1.2-3: Cell surface EGFR expression (MFI) in colon cancer cell lines correlates negatively with EGF-SubA sensitivity.

Dot plot created using the IC₅₀ obtained from the EGF-SubA dose response curves and mean fluorescent intensity (MFI) of PE-conjugated anti-EGFR antibody using flow cytometry for a panel of colon cancer cell lines. (IC₅₀ = inhibitory concentration required to cause 50% growth inhibition at day 4). Pearson's correlation coefficient = -0.83

Table 4.1-1: Cell surface EGFR expression (MFI) in colon cancer cell lines correlates negatively with EGF-SubA sensitivity (IC₅₀).

Cell line	MFI	IC ₅₀ EGFSubA/pM
SW620	7.14	550
HCT116p53wt	21.37	75
Lovo	22.16	45
HT29	27.72	35
HCT116p53null	33.91	55*
SW480	38.36	25

Rho=-1.0 (P=0.046); *excluded from Spearman rank correlation analysis

4.1.3 EGF-SubA activates EGFR trafficking

Ligand binding leads to rapid internalisation of EGFR within minutes.(363, 364) Proteosomes are implicated in the regulation of EGFR endocytosis and proteosome inhibitors such as MG132 can reduce EGFR degradation.(365) Internalised ligand bound EGFR complexes are delivered to early endosomes where they are trafficked for recycling or degradation. For degradation, EGFRs are transported to prelysosomal late endosomes then degraded by lysosomes. This EGF-induced down-regulation of EGFR is a major negative feedback regulatory mechanism that controls the density and duration of receptor signalling.(366)

By demonstrating trafficking of the EGFR following treatment with EGF-SubA, it can be concluded that the EGF moiety is functionally active. An initial pilot western blot demonstrated that EGFR was down-regulated following exposure to EGF-SubA (Figure 4.1.3-1).

I hypothesised that if the observed reduction in EGFR following treatment with EGF-SubA is due to internalisation and lysosomal degradation via complexing with the EGF moiety, then it should be possible to inhibit this process using proteosome inhibitors such as MG132 or Leupeptin.

Indeed, when cells were exposed to EGF-SubA in the presence of MG132, EGFR expression is rescued. Leupeptin seemed less active in this experiment. Treatment with proteosome inhibitors does not however abrogate GRP78 cleavage (Figure 4.1.3-2).

This data is in keeping with that of Backer et al., who confirmed that EGF-SubA was able to induce EGFR-tyrosine autophosphorylation at comparable concentrations to recombinant EGF.(314) In my experiment, it can be observed that while both

recombinant EGF and EGF-SubA cause reduced levels of EGFR, only EGF-SubA causes cleavage of GRP78. Again, Backer concluded that EGFR-mediated endocytosis was critical for delivery of EGF-SubA into cells following the observation that GRP78 cleavage was significantly delayed using untargeted SubA.(314)

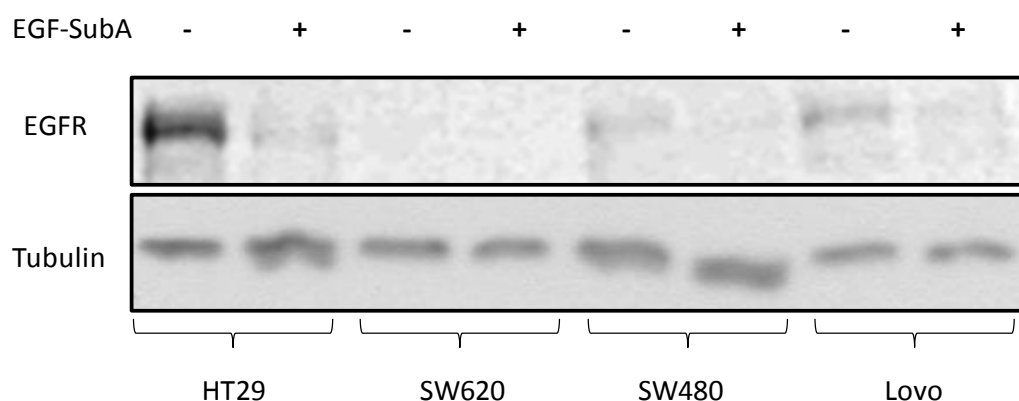


Figure 4.1.3-1: EGFR expression is reduced following exposure to EGF-SubA.

Cells were seeded into six-well plates at 30% confluence in standard media which was changed to low serum (1%) after 6hrs to enhance EGFR expression then incubated for 24hr prior to treatment with 1nM EGF-SubA. Cells were harvested after 24hr for western blot.

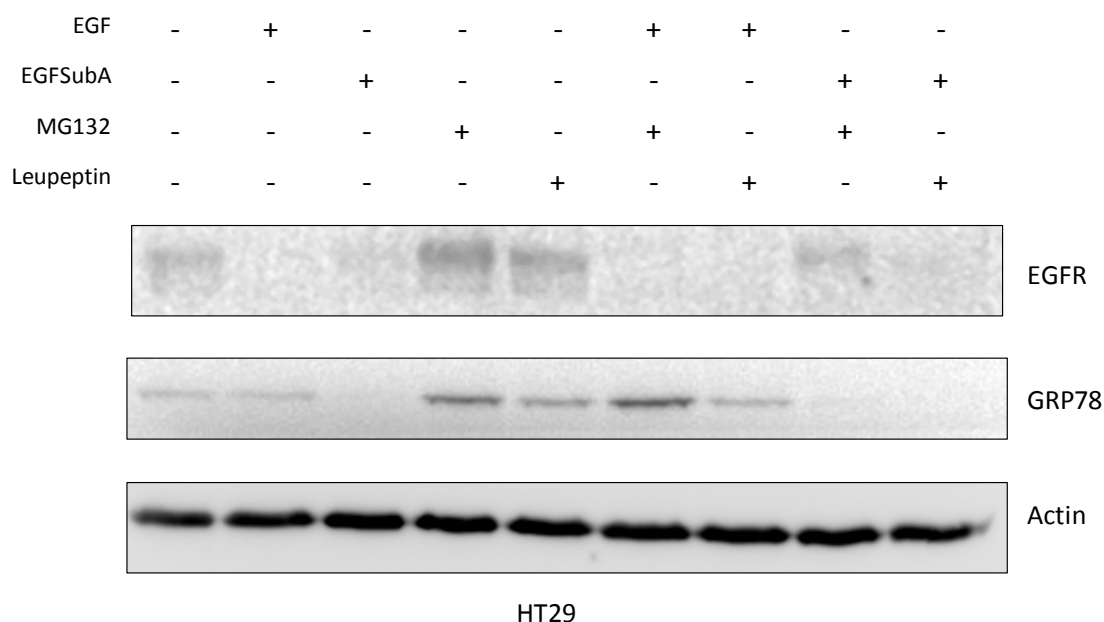


Figure 4.1.3-2: Proteasome inhibitor MG132 rescues EGFR expression following exposure to EGF-SubA, but does not prevent cleavage of GRP78.

Cells were seeded into six-well plates at 30% confluence in standard media which was changed to low serum (1%) after 6hrs to enhance EGFR expression then incubated for 24hrs prior to treatment. For combination treatment, Leupeptin (25 µg/ml) or MG132 (100µM) were added 1 hr prior to EGF (100 ng/ml) or EGF-SubA (1 nM). Treatments were terminated at 4hrs by exchanging media and cells were then harvested for western blot.

4.1.4 The EGFR-mediated proteolytic activity of EGF-SubA is dose-dependent.

Having demonstrated that EGF-SubA cytotoxicity correlates with EGFR, I evaluated if cleavage of GRP78 was also defined by EGFR expression. Western blot using an antibody specific for GRP78 (sc13968) confirmed EGF-SubA cleavage of GRP78 revealing a 28 kDa fragment. Equivalent amounts of EGF-SubA were unable to induce significant cleavage of GRP78 in SW620 cells (EGFR-negative) compared with the other cell lines analysed as shown below in Figure 4.1.4-1.

Interestingly, it was observed that all cell lines, including SW620 cells, respond to continuous EGF-SubA exposure by upregulating GRP78 expression. This can be noted in Figure 4.1.4-1 whereby the band density of intact GRP78 is seen to increase up to a dose of 100 pM for HCT116p53wt, HCT116p53null and HT29 cells; up to 60 pM in the sensitive SW480 cells, and even at 1 nM in SW620. At higher doses, the amount of GRP78 cleavage by EGF-SubA seems to overwhelm this compensatory mechanism and the amount of intact GRP78 is reduced.

To examine the kinetics of the EGFR-mediated proteolytic activity of EGF-SubA, HCT116p53wt cells were selected for a time course experiment. Apparent as early as 45 minutes to 1.5 hours following exposure to EGF-SubA, a substantial decrease in intact GRP78 is observed by 3 hours but by 24 hours of continuous GRP78 cleavage, HCT116p53wt cells respond by upregulating GRP78 (Figure 4.1.4-2). Similarly, a cleaved 28 kDa fragment of GRP78 can be detected by 1.5 hours. One must acknowledge that interpretation of GRP78 protein levels may be complicated by the SubA mediated cleavage.

It is known that selective cleavage of GRP78 by SubA toxin releases PERK, IRE1 and ATF6 leading to overwhelming activation of the UPR and subsequent apoptosis (312, 313). Therefore, upregulation of GRP78 is likely to be secondary to activation of the unfolded protein response, which is a slow compensatory mechanism (>24 hours) compared with the rapid (minutes) cleavage of GRP78 as observed in HCT116p53wt cells.

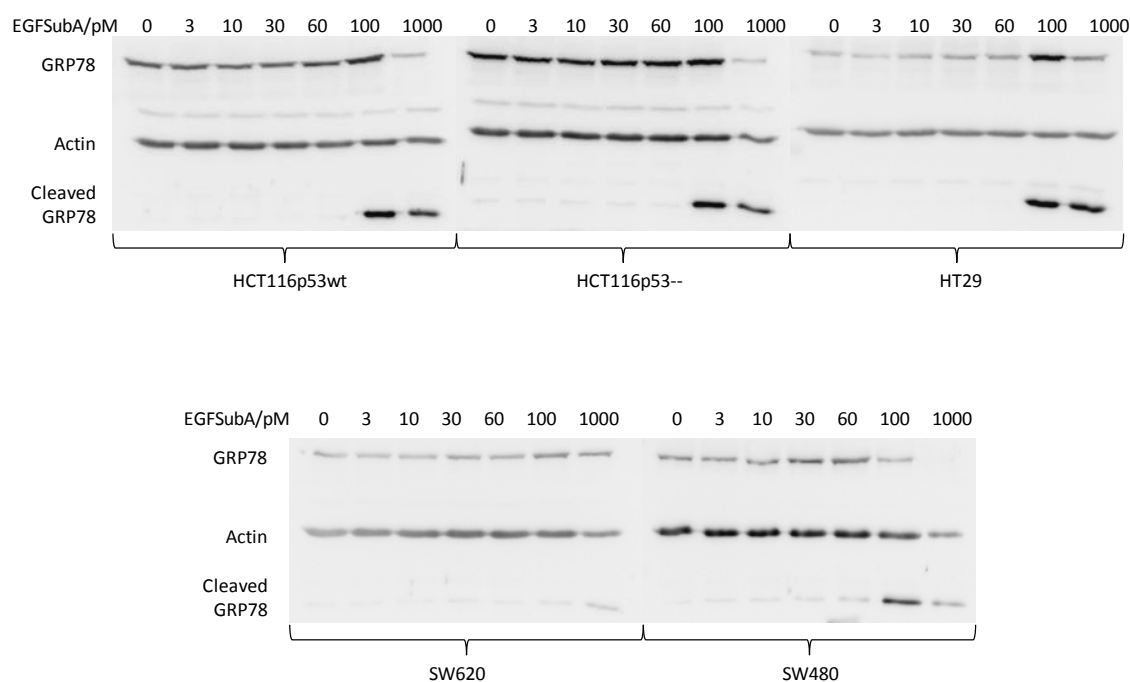


Figure 4.1.4-1: Western blot demonstrating EGF-SubA mediated cleavage of GRP78.

Cells were seeded into six-well plates and 24hr later exposed to varying doses of EGF-SubA or vehicle control as indicated. After 24hr cells were harvested, and protein extracts were subject to western blotting to demonstrate GRP78 cleavage. Actin was used as a loading control.

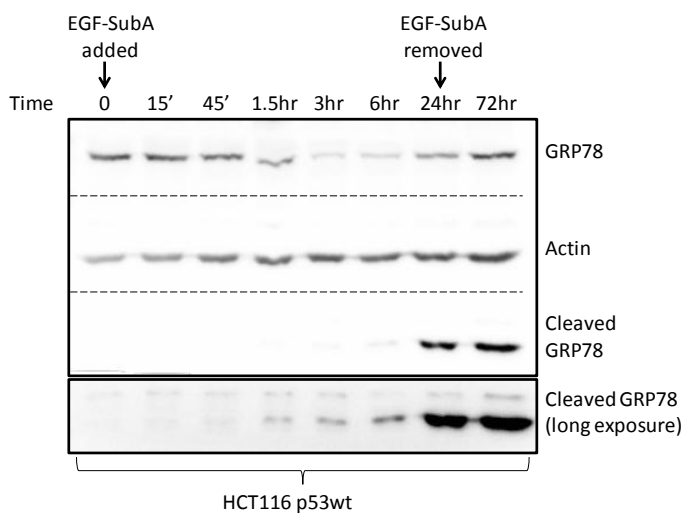


Figure 4.1.4-2: EGF-SubA causes rapid proteolytic cleavage of GRP78.

HCT116p53wt cells were seeded on to 10cm plates. After 24 hr 1 nM EGF-SubA was added and cells harvested at varying time points as indicated, and protein extracts subjected to western blotting.

4.1.5 Activation of the unfolded protein response by EGF-SubA leads to cell cycle arrest.

As discussed above, rapid GRP78 cleavage results in release of the UPR effectors PERK, IRE1 and ATF6. To examine the downstream effects of EGF-SubA-mediated cleavage of GRP78, I examined for the well described UPR target C/EBP homologous protein (CHOP), also known as GADD153, a known pro-apoptotic factor (as discussed in section 1.3.3.3.1 on page 59).(261)

ER stress can induce transcription of CHOP via multiple UPR pathways including ATF4, spliced ATF6, and XBP-1 derived from spliced Xbp1 mRNA.(367) CHOP induction as a result of GRP78 cleavage has been previously documented,(311, 313) and is indicative a severe ER stress. Although known for its pro-apoptotic effects, CHOP is also implicated in causing G1/S-phase cell cycle arrest.(368) This is of further interest when it is also considered that SubAB-treated Vero and HeLa cells are known to arrest in G1 phase, and this is due to cyclin D1 downregulation or proteosomal degradation.(369)

In my experiments, evidence of UPR activation following exposure to EGF-SubA indicated by compensatory upregulation of GRP78 and increased expression of CHOP, and the observation that EGF-SubA inhibits cell proliferation, led me to believe that cellular responses to EGF-SubA were likely to be due to cell cycle arrest. To investigate this I performed cell cycle analysis with Propidium iodide using flow cytometry as described in section 2.7.2.

An initial analysis of HCT116p53wt cells demonstrated that greater than 80% of cells were in G1 after treatment with EGF-SubA; in contrast 69% of untreated cells were in G1 (

Figure 4.1.5-2: EGF-SubA induces G1 cell cycle arrest.).

Although measuring the DNA content of cells using propidium iodide is a useful way of measuring changes in the cell cycle, the S-phase cells cannot be accurately determined using this method. A more accurate analysis is allowed by performing S-phase analysis using bromodeoxyuridine uptake. As discussed in section 2.7.3, the determination of the percentage of cells in S-phase is dependent upon the detection of a thymidine analogue, bromodeoxyuridine (BrdU), which when added to culture medium is incorporated into DNA during DNA replication. Via flow cytometry, a bivariate analysis is performed of total DNA content using propidium iodide staining along the X-axis plotted against BrdU incorporation detected via FITC labelled antibody to BrdU on the Y-axis. Only those cells that have been actively synthesising DNA during the time that BrdU is present will be positive for it. Simultaneous staining with propidium iodide allows the DNA content to be assessed and thus, the percentage of cells in G1, S-phase and G2/M can be determined.

As predicted, treatment with EGF-SubA resulted in a reduction in S-phase with arrest in G1. Only the EGFR-negative cell line SW620 was resistant to this effect with minimal change in cytogenetics (Figure 4.1.5-3, Figure 4.1.5-4, Table 4.1-2).

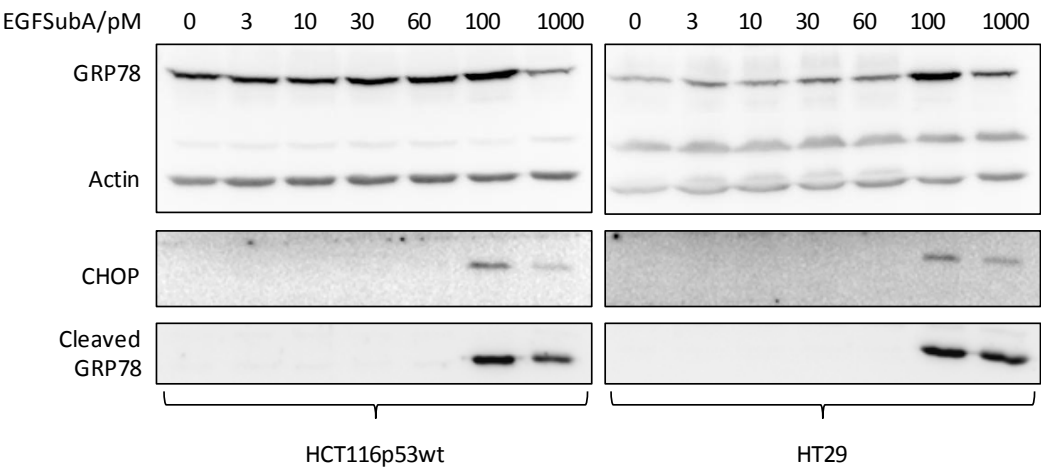


Figure 4.1.5-1: GRP78 cleavage induces transcription of the stress-inducible CHOP.

Cells were treated essentially as in Figure 4.1.4-1. Cells were seeded into six-well plates and 24hr later exposed to varying doses of EGF-SubA or vehicle control as indicated. After 24hr cells were harvested, and protein extracts were subject to western blotting. Actin was used as a loading control.

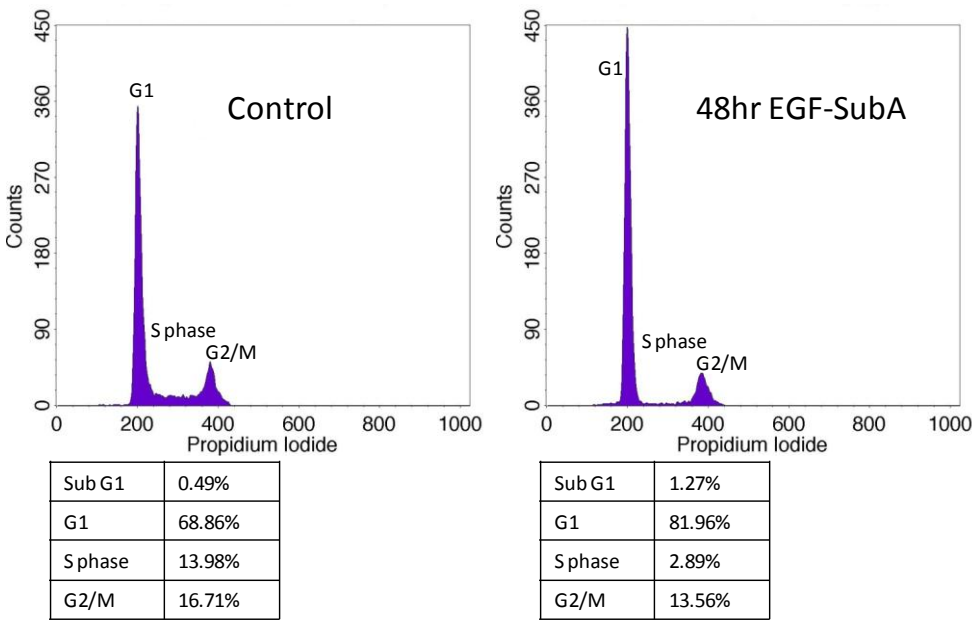


Figure 4.1.5-2: EGF-SubA induces G1 cell cycle arrest.

HCT116p53wt cells were seeded into six-well plates and after 24 hr were treated with 1 nM EGF-SubA or vehicle control. After 48 hr, cell cycle analysis was performed on fixed permeabilised cells stained with Propidium iodide.

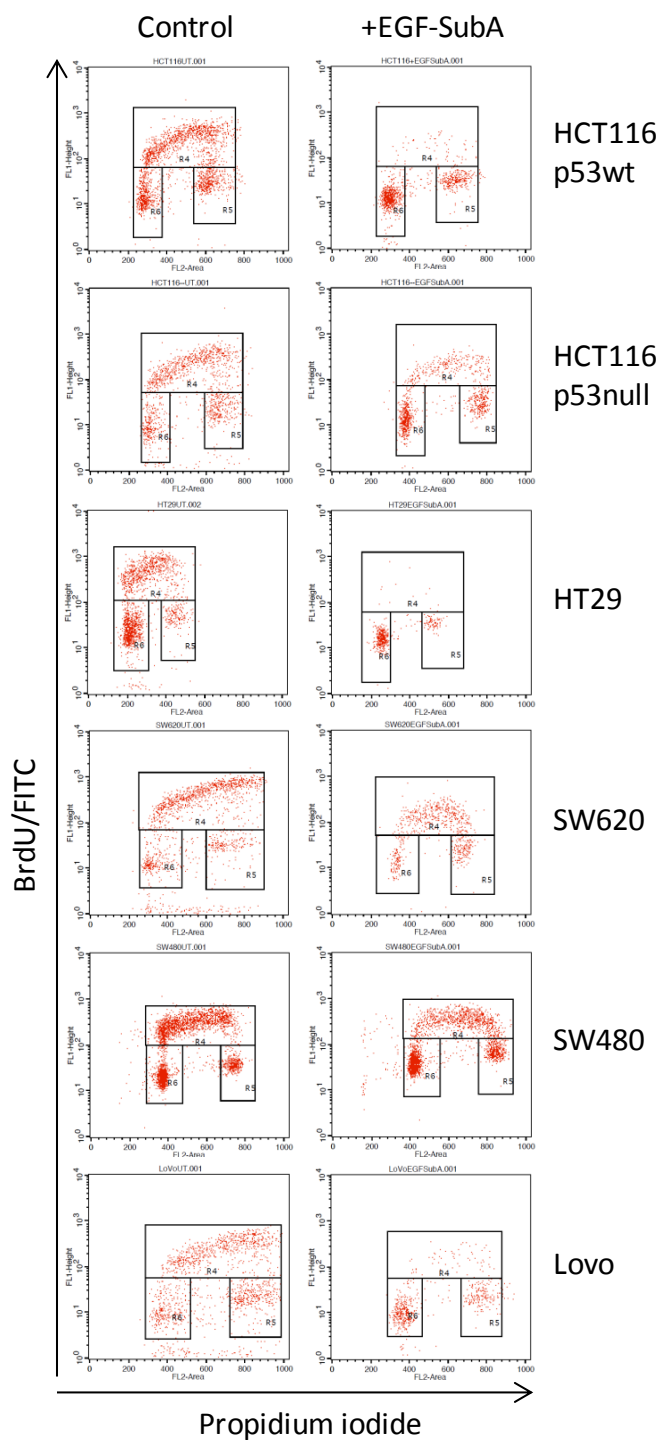


Figure 4.1.5-3: Flow cytometric cell cycle analysis of BrdU incorporation demonstrates G1 arrest following exposure to EGF-SubA.

Cells were seeded in six-well plates and after 24 hr exposed to a dose equivalent to 3 x IC₅₀ of EGF-SubA for a further 24 hr. Cells were treated with 10 μ M of BrdU for 60 mins prior to harvesting for flow cytometry as described in section 2.7.3.

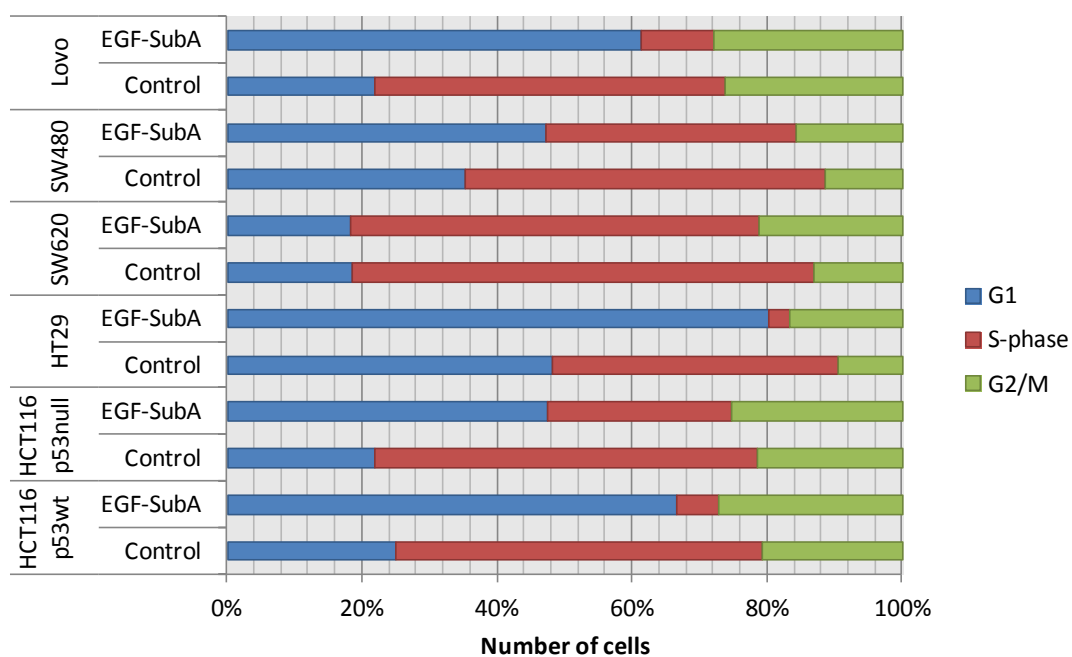


Figure 4.1.5-4: Histogram representing the effect on the cell cycle of exposure to EGF-SubA.

Data extrapolated from BrdU assay in Figure 4.1.5-3.

Table 4.1-2: Table demonstrating the percentage reduction of cells in S-phase following exposure to EGF-SubA compared with controls.

Data extrapolated from BrdU assay in Figure 4.1.5-3.

Cell line	% Reduction in S-phase after EGF-SubA
HT29	93
HCT116p53wt	89
Lovo	78
HCT116p53null	50
SW480	32
SW620	5

4.1.6 EGF-SubA is a weak inducer of apoptotic cell death

I have shown that EGF-SubA can produce growth inhibition and cell cycle arrest in colon cancer cells at picomolar concentrations. Western blotting of samples incubated with EGF-SubA has revealed that EGF-SubA is a potent inducer of ER stress, as indicated by a rapid upregulation of GRP78 and induction of CHOP. As discussed in the introduction to this thesis, severe or prolonged ER stress can lead to apoptosis. The ability of EGF-SubA to induce CHOP, a known proapoptotic factor would suggest that EGF-SubA may be able to cause apoptosis.

PARP (poly(ADP-ribose) polymerase) is an abundant nuclear protein with important functions in DNA repair in response to numerous cytotoxic insults. Caspase-mediated cleavage of PARP is a useful indicator of apoptotic cell death (as oppose to necrotic death) and reflects irreversible cell damage and commitment to the apoptotic pathway.(370, 371) Despite the apparent potency of EGF-SubA as an inhibitor to cancer cell proliferation, western blot revealed that EGF-SubA was not a potent inducer of PARP cleavage (Figure 4.1.6-1), even at a dose far above that required to inhibit growth (1 nM).

As the above experiment used cells treated for 24 hours with 1 nM EGF-SubA, I treated cells with an even higher dose of 2 nM and assessed for apoptotic cell death at 24 hour intervals to see if this higher dose could induce apoptosis over a longer period of time. Figure 4.1.6-2 demonstrates that only a modest amount of apoptosis could be detected by annexin V assay (20% increase compared with control), and this was not apparent until 48 hours of treatment with no further increase at 72 hours. (For comparison, one can compare the marked apoptosis resulting from combination treatment with 5-FU and oxaliplatin later in Figure 4.1.8-3.) Analysis of the data

reveals a small increase in early apoptosis (PI–ve/AV+ve, lower right quadrant on bivariate analysis) at 24 hours that increases by 48 hours and by 72 hours, most apoptotic cells are demonstrating late apoptosis (PI+ve/AV+ve, upper right quadrant), with a consequent reduction of early apoptosis. It is notable that the amount of necrotic cell death (PI+ve/AV–ve, upper left quadrant) is minimal or zero for all conditions.

Thus it can be concluded that in contrast to its potent inhibitory effect on proliferation in colon cancer cells, EGF-SubA is only a weak inducer of apoptosis.

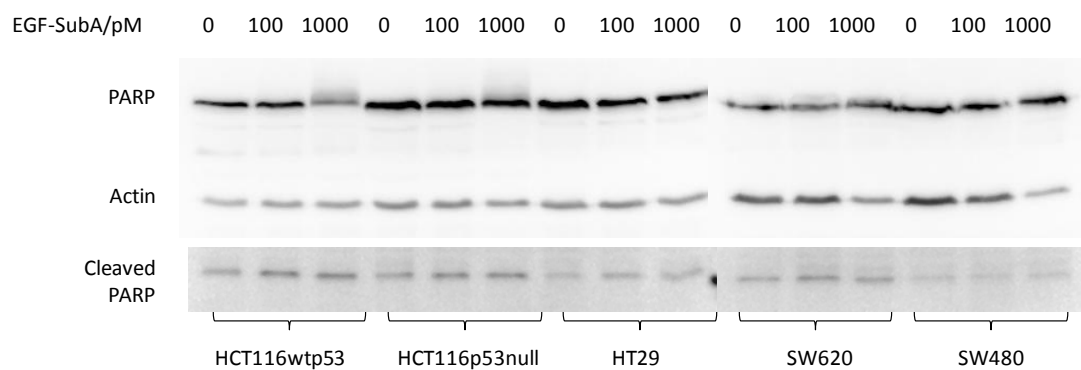


Figure 4.1.6-1: EGF-SubA is not a strong inducer of PARP cleavage.

A panel of colon cancer cells were incubated in 6 well plates with varying doses of EGF-SubA for 24 hr prior to harvest. Cell lysates were subject to SDS-PAGE using 15% polyacrylamide gel for separation prior to immunoblotting with mouse monoclonal anti-PARP antibody (BD Transduction) that recognises both the uncleaved and cleaved forms of PARP. Actin was used as a loading control.

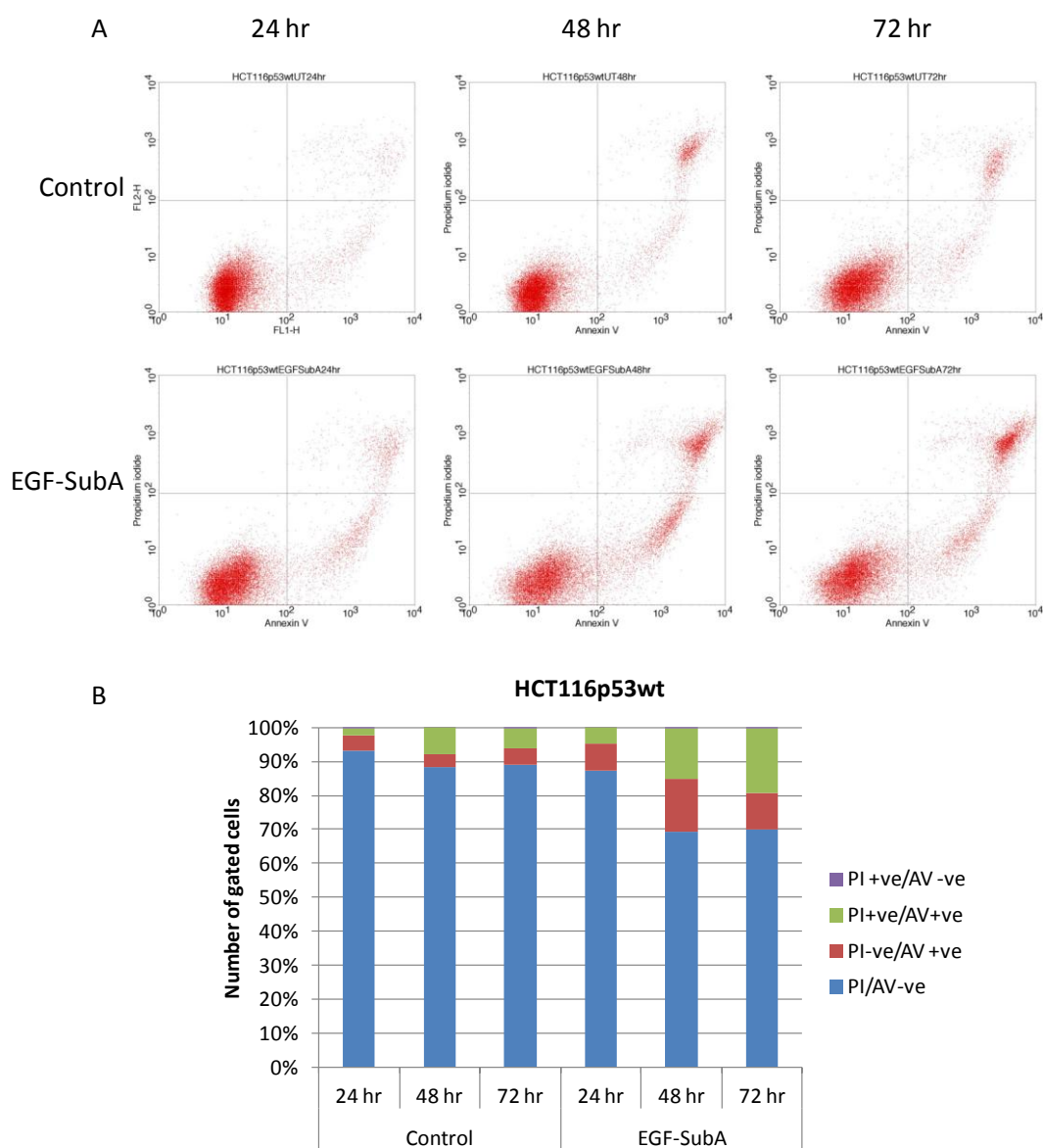


Figure 4.1.6-2: EGF-SubA is a weak inducer of apoptosis.

HCT116p53wt cells were seeded to 10cm plates and exposed to 2 nM EGF-SubA or vehicle control for 24, 48 and 72 hr. At each timepoint, cells were harvested analysed by flow cytometry. (A) Bivariate distributions of Annexin V (AV)-positivity (apoptosis, FL1 parameter, x-axis) vs. Propidium iodide (PI)-positivity (necrosis, FL2 parameter, y-axis) were generated for each population. Viable cells were measured as those in the lower left quadrant (PI/AV –ve); early apoptotic cells are in the right lower quadrant (PI –ve/AV +ve, late apoptotic cells are in the right upper quadrant (PI +ve/AV +ve); remaining cells in the left upper quadrant were deemed necrotic or dead (PI +ve/AV –ve). (B) These analyses were represented by histogram.

4.1.7 EGF-SubA and conventional drug treatment of colorectal cancer

I have observed that EGF-SubA may cause cell cycle arrest and inhibit proliferation of colon cancer cell lines at picomolar concentrations. This raises the important question of whether EGF-SubA may have a potential role in the drug treatment of colorectal cancer. As discussed earlier, the mainstay of treatment of colorectal cancer, in both the adjuvant and metastatic setting, is the combination of 5-FU/FA and Oxaliplatin.

EGF-SubA has been shown to synergise with the ER-stress inducing drug thapsigargin.(314) Thapsigargin inhibits ER calcium-dependent ATPase, thus depleting ER calcium stores and impairing chaperone function, leading to increased unfolded proteins and consequently activating the UPR. I could find no evidence that either 5-FU or oxaliplatin are known to induce ER-stress. Therefore, I would not expect either drug to synergise with EGF-SubA, however, as these drugs exhibit common systemic toxicity, any additive effect that allows a reduction of dose may still be relevant.

Combination assay of EGF-SubA and Oxaliplatin performed on HT29 and HCT116p53wt cells demonstrated some evidence of synergy with combination indices (CI) of less than 1 for one data point on the ED₅₀ Isobologram in each cell line, however, in the case of HCT116 cells, it was possible to deduce two further data points both of which gave a CI of 1 indicating an additive effect (Figure 4.1.7-3).(372)

Combination assay of EGF-SubA and 5-FU revealed two data points with a CI of less than 1 suggesting synergy, whilst two further data points gave a CI equal to 1 indicating an additive effect (Figure 4.1.7-6).

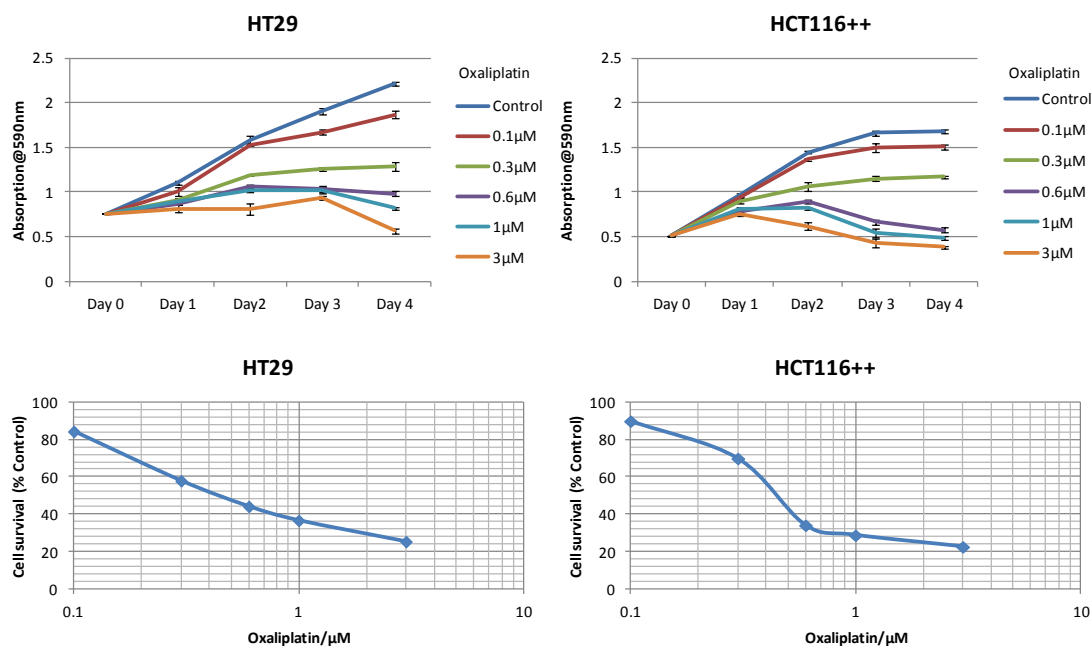


Figure 4.1.7-1: Proliferation and dose response curves for Oxaliplatin.

HT29 and HCT116p53wt were seeded onto 96 well plates at a concentration of 3000 cells/well., and treated with varying concentrations of Oxaliplatin as shown or vehicle control. Drugs and media were changed daily. MTT assay was performed daily. Error bars represent the s.e.m. from four duplicate wells. Dose response curves were calculated from day 4 data to calculate the IC_{50} dose.

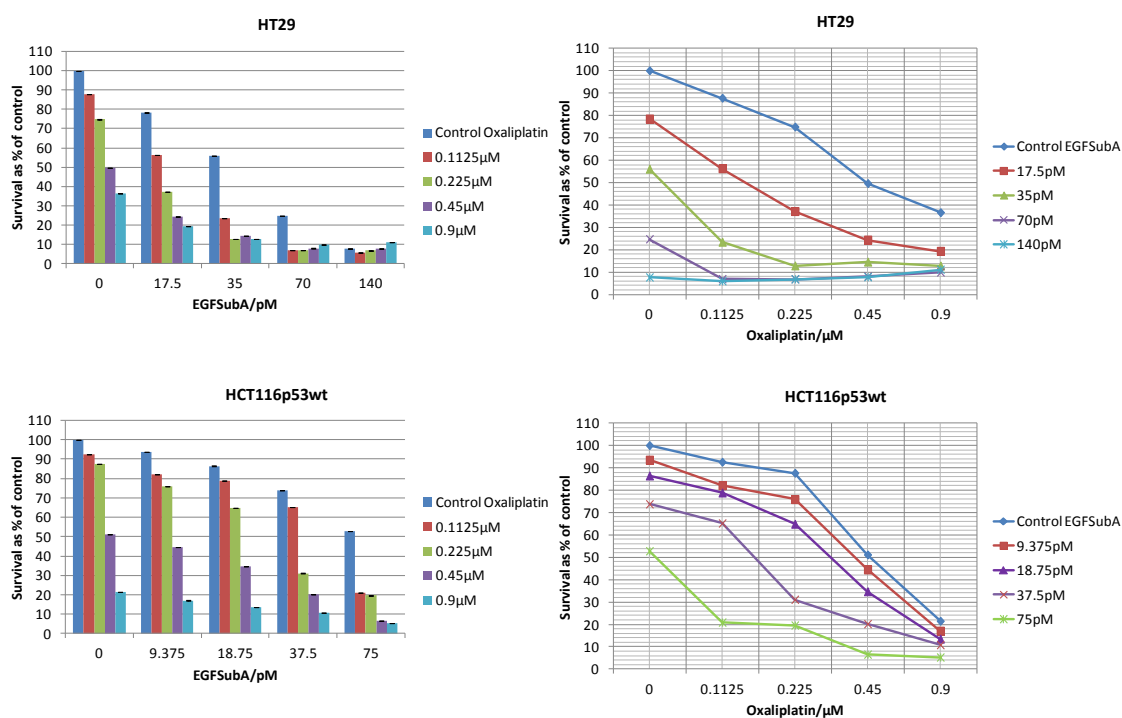
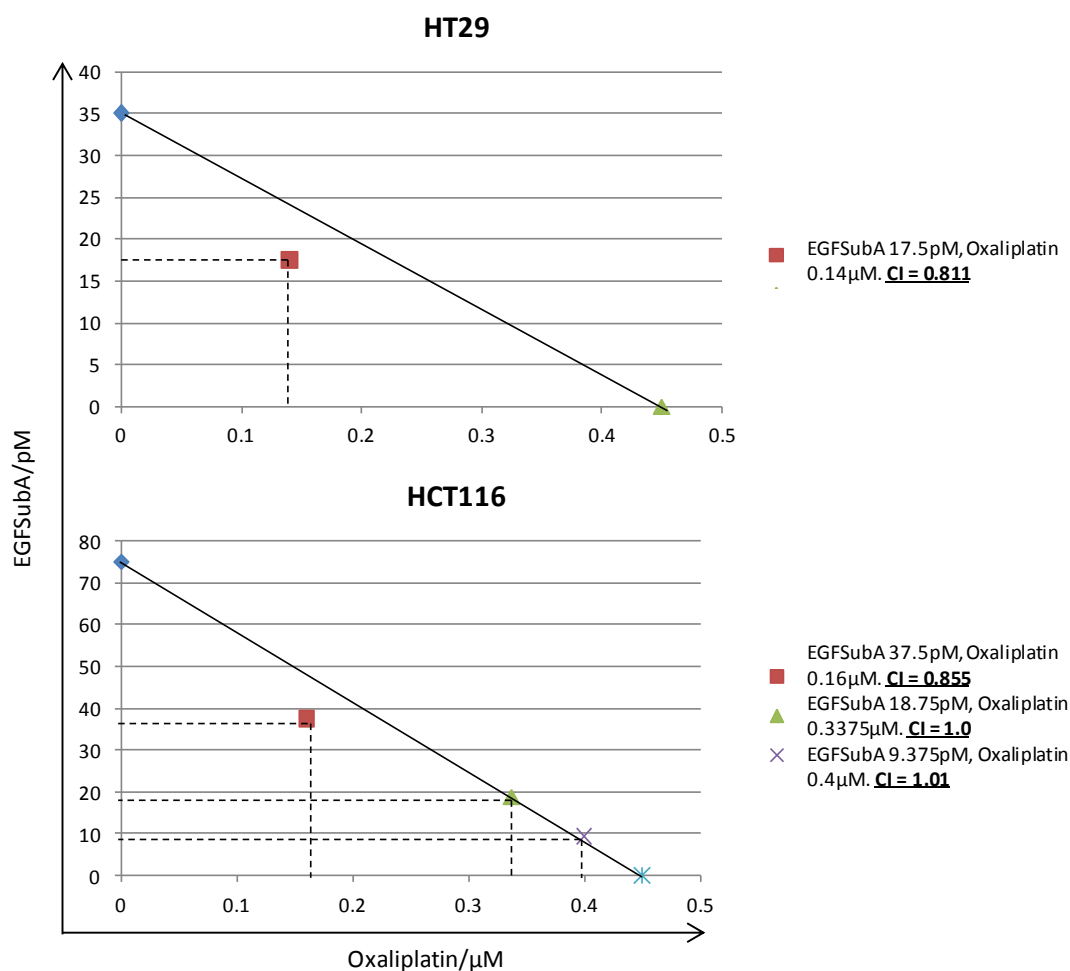


Figure 4.1.7-2: EGF-SubA in combination with Oxaliplatin.

Cells were seeded into 96 well plates as previously and treated with varying doses of EGF-SubA and/or Oxaliplatin. Drugs and media were changed daily. MTT assay was performed on day 4. The histograms represent % survival compared with control with error bars showing the s.e.m. from four duplicate wells. The dose response curves to the right were used to calculate combination IC_{50} doses.



CI < 1 Synergism

CI = $D_1/(Dx)_1 + D_2/(Dx)_2$ CI = 1 Additive effect

CI > 1 Antagonism

Figure 4.1.7-3: ED₅₀ Isobologram for EGFSuBA and Oxaliplatin.

The Isobologram demonstrates the data points for each combination of doses that produce an IC₅₀ as determined from the dose response curves. If the data point falls on the hypotenuse (solid black oblique line connecting each individual IC₅₀), an additive effect is indicated. If the data points fall on the lower left, or on the upper right, synergism or antagonism are indicated, respectively. The combination index (CI) is indicated for each data point, and is calculated using the formula demonstrated where (Dx)₁ and (Dx)₂ is the dose of each drugs which, when used alone produces a fixed affect (in this case the IC₅₀). The numerators D₁ and D₂ are the doses of each drug which when used in combination, achieve the same fixed effect.

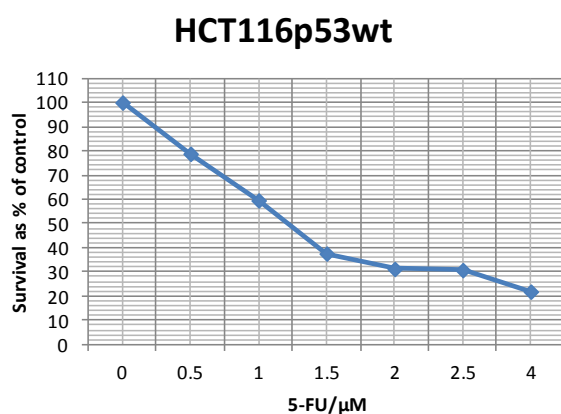


Figure 4.1.7-4: Dose response curve for 5-FU.

HCT116p53wt cells were seeded into a 96 well plate. After 24 hr, 5-FU was added in varying doses as indicated.

Drugs and media were changed daily. MTT assay was performed after 4 days. Error bars are present but are too narrow to be visible on the figure.

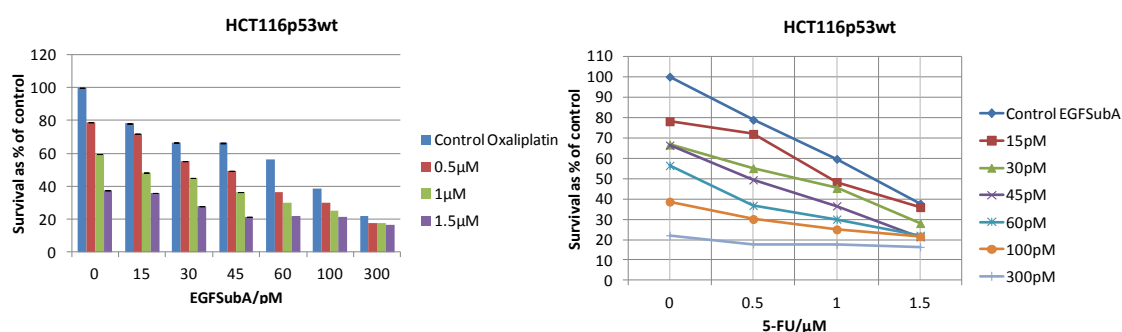


Figure 4.1.7-5: Combination of EGF-SubA and 5-FU.

Performed essentially as previously for EGF-SubA and Oxaliplatin. Cells were seeded into 96 well plates as previously and treated with varying doses of EGF-SubA and/or 5-FU. Drugs and media were changed daily. MTT assay was performed on day 4. The histograms represent % survival compared with control with error bars showing the s.e.m. from four duplicate wells. The dose response curves to the right were used to calculate combination IC_{50} doses.

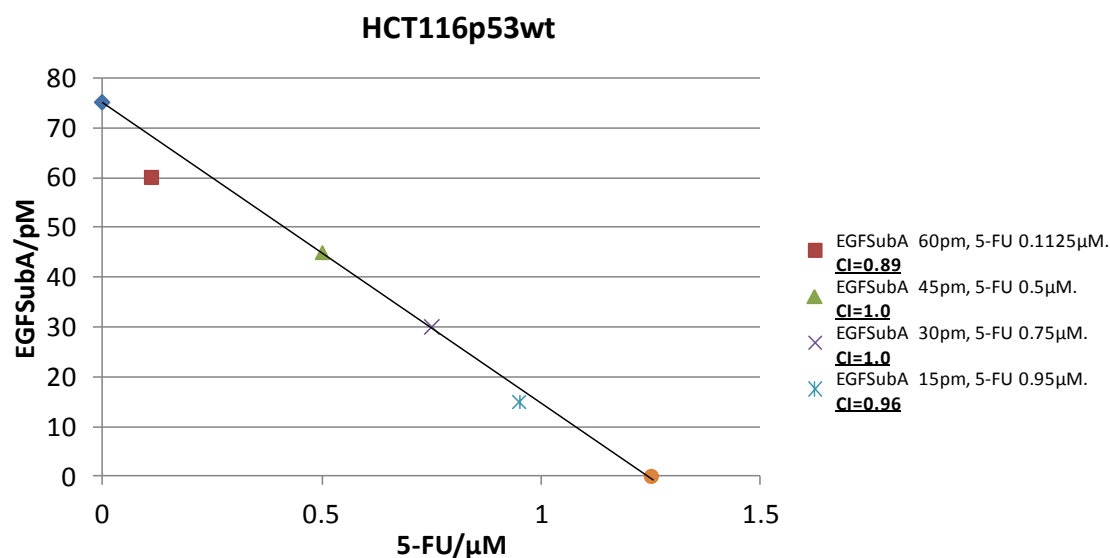


Figure 4.1.7-6: ED₅₀ Isobologram for combination treatment of HCT116p53wt cells with EGFSuBA and 5-FU.

Isobologram constructed in the same manner as Figure 4.1.7-3. Combination IC₅₀ points were extrapolated from the previous figure. Combination indices are indicated for each data point.

4.1.8 EGF-SubA reduces the apoptotic response to 5-FU and Oxaliplatin

It is clear that EGF-SubA can induce cell cycle arrest, and may have an additive or mildly synergistic effect upon reducing proliferation when combined with 5-FU or Oxaliplatin, as measured by MTT assay in the previous experiment. I was interested to establish if this effect could be attributed to increase in apoptosis.

Using HCT116p53wt cells, I performed an Annexin V assay using flow cytometry to assess relative apoptosis following treatment with EGF-SubA with or without 5-FU. High doses of each drug were used in order to obtain measurable degrees of apoptosis over a period of 24 hours. Cells cultured for each condition were also retained for staining with propidium iodide for cell cycle analysis.

As a single agent, even at a high dose of 2 nM, EGF-SubA did not result in significant apoptotic cell death at 24 hr of treatment. In a striking comparison to the additive effect seen in the combination data obtained using the MTT assay, there was a significant reduction in apoptotic cell death when EGF-SubA was combined with 5-FU (24% reduction) compared with treatment with 5-FU alone (Figure 4.1.8-1). Minimal necrotic cell death was seen with either treatment at 24 hours.

5-FU is an antimetabolite drug whose cytotoxicity results from the misincorporation of its active metabolites into RNA and DNA and its inhibition of thymidylate synthase, which is necessary for DNA replication and repair.⁽⁷³⁾ Thus it is most active against cells replicating their DNA in S-phase of the cell cycle.

I performed cell cycle analysis to determine if the effects of EGF-SubA upon 5-FU described above might be explained by G1 arrest and a concomitant reduction of cells entering S-phase where they are most susceptible to treatment with 5-FU. As shown in Figure 4.1.8-2, 24 hours of 5-FU treatment resulted in the accumulation of

cells in late G1 and S-phase, a concurrent loss of G2 cells, as well as a significant increase of cells in the sub G1 apoptotic phase, consistent with previous reports. (342-344) In earlier observations, treatment with EGF-SubA resulted in the majority of cells accumulating in G1. By way of contrast, following exposure to both drugs in combination, the majority of cells remain in G1, however, the number of cells in S-phase was higher compared with control, or single agent EGF-SubA treated cells. There were also significantly fewer cells in the sub G1 phase compared with 5-FU treated cells.

Together, these profiles characterise the differences in cell cycle progression that may underlie the observed reduction in apoptosis seen when EGF-SubA is combined with 5-FU. It is likely that the arrest of cells in the G1 phase reduces the number of cells that, when treated with 5-FU, can progress into S-phase and undergo apoptosis. These findings also raise a question regarding the role of GRP78 in sensitivity to 5-FU. EGF-SubA causes highly specific cleavage of GRP78 and it is possible that an acute reduction in cellular GRP78 following exposure to EGF-SubA, whether it be within the endoplasmic reticulum or elsewhere within the cell, may somehow affect the apoptotic response to 5-FU. This argument is strengthened by the analysis of the clinical data presented earlier in this thesis where I demonstrated that patients whose tumours weakly expressed GRP78 failed to respond to 5-FU based adjuvant chemotherapy, whereas those with elevated GRP78 had a good response (see section 3.1.8). It is also interesting to contrast the effects of GRP78 depletion by EGF-SubA with that caused by transient transfection with siRNA targeting GRP78 (see section 3.1.11). In both cases, depletion of GRP78 led to a reduced effect of 5-FU treatment in vitro; however, siRNA transfection did not lead to any major correction of the cell

cycle, unlike EGF-SubA which led to G1 arrest. Both these data add to the argument that GRP78 may have an, as yet undetermined, functional role in the response to 5-FU. This may include effects mediated by GRP78 residing outside of the endoplasmic reticulum that may affect pathways other than the unfolded protein response.(373)

As contemporary drug treatment for colorectal cancer relies upon the combination of 5-FU/FA and Oxaliplatin (as discussed in section 1.1.8.3), I proceeded to examine apoptotic response to this combination and the effects of adding EGF-SubA. As Figure 4.1.8-3 demonstrates, Oxaliplatin as a single agent is not particularly effective at inducing apoptosis (14% increase in apoptosis), even at the high non-clinically relevant doses used in this experiment. However, in combination with 5-FU, a dramatic apoptotic response (74% increase apoptosis) is seen in the HCT116p53wt cells. This is consistent with the synergism observed in preclinical studies of 5-FU and Oxaliplatin,(374) and the subsequent confirmation by phase III trials that show significant improved response rates and survival for the combination.(79) As in the previous experiment, the response to 5-FU as a single agent is reduced when in combination with EGF-SubA (15% reduction compared with control). Similarly, the response to Oxaliplatin is also reduced by EGF-SubA but to a lesser degree (3% reduction compared with control). When EGF-SubA was combined with 5-FU+Oxaliplatin, a large reduction in apoptotic events (46% reduction) was seen compared with the combination of 5-FU and Oxaliplatin alone.

The cell cycle profiles for the combination experiment with 5-FU, Oxaliplatin and EGF-SubA are shown in Figure 4.1.8-4. When HCT116p53wt cell are treated with Oxaliplatin for 24 hours, the majority of cells arrest in G2/M (20% increase

compared with control). There is also a small increase of cells in sub G1 (2% increase). The cell cycle profile after treatment with 5-FU is similar to the previous experiment. The combination of the two drugs led to the majority of cells arresting in S-phase, similar to treatment with 5-FU alone, however a greater number of cells are seen in sub G1 (9% v. 3%, for 5-FU and Oxaliplatin compared with 5-FU alone, respectively). The addition of EGF-SubA to Oxaliplatin attenuates the G2/M arrest. In combination with 5-FU and Oxaliplatin, EGF-SubA led to a reduction in S-phase arrest, with more cells residing in G1 and G2/M phases and a reduction of apoptotic sub G1 cells.

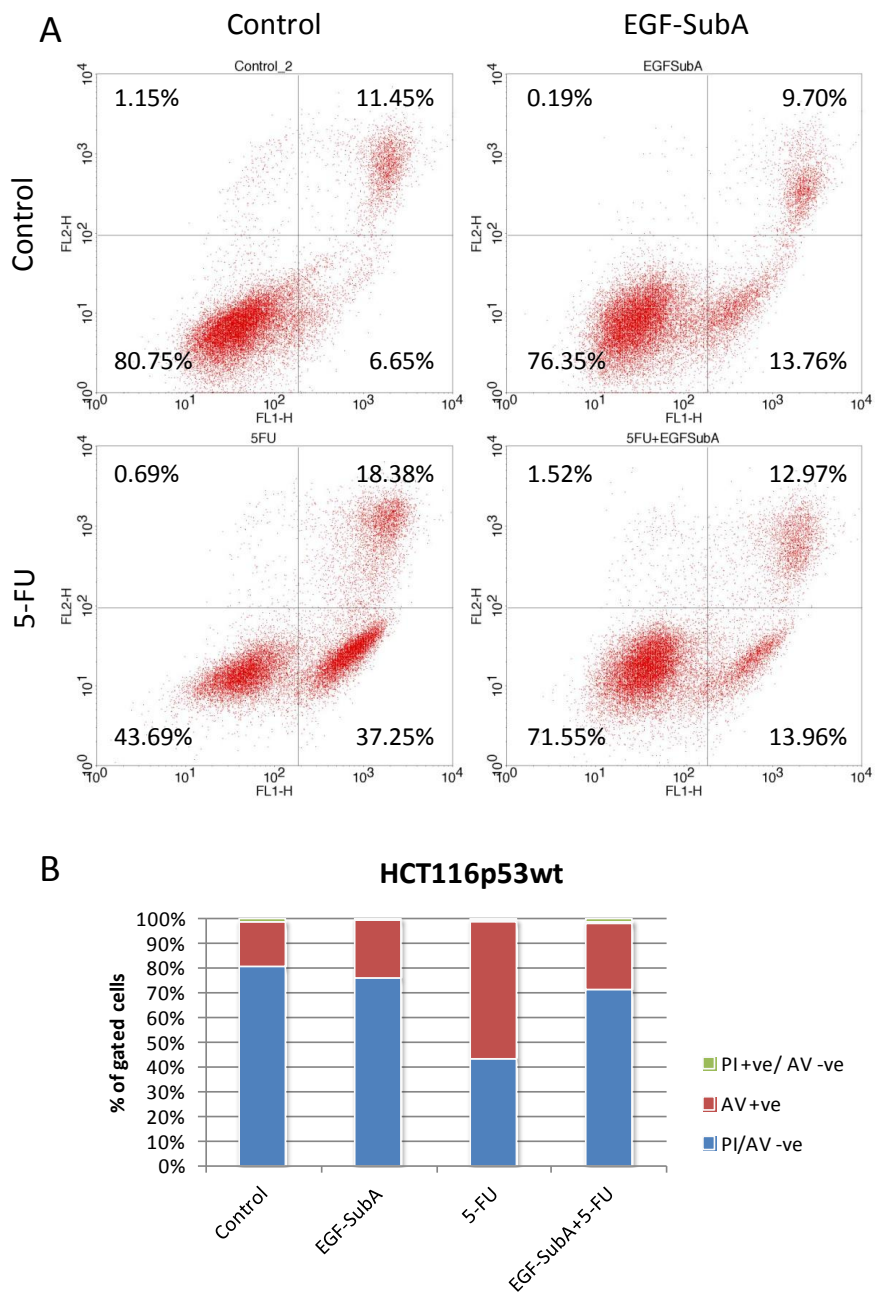


Figure 4.1.8-1: EGF-SubA attenuates the apoptotic response to 5-FU.

(A) HCT116p53wt cells were incubated with EGF-SubA (2 nM) and/or 5-FU (380 μ M), or vehicle control (PBS). After 24 hr of incubation cells were harvested for Annexin V assay. (A) Bivariate distributions of Annexin V (AV)-positivity (apoptosis, FL1 parameter, x-axis) vs. Propidium iodide (PI)-positivity (necrosis, FL2 parameter, y-axis) were generated for each population. Viable cells were measured as those in the lower left quadrant (PI/AV -ve); early apoptotic cells are in the right lower quadrant (PI -ve/AV +ve, late apoptotic cells are in the right upper quadrant (PI +ve/AV +ve); remaining cells in the left upper quadrant were deemed necrotic or dead (PI +ve/AV -ve). (B) These analyses were used to generate percentages of viable (PI/AV -ve), apoptotic (AV +ve) or dead (PI +ve/AV -ve) cells at 24 hr of treatment.

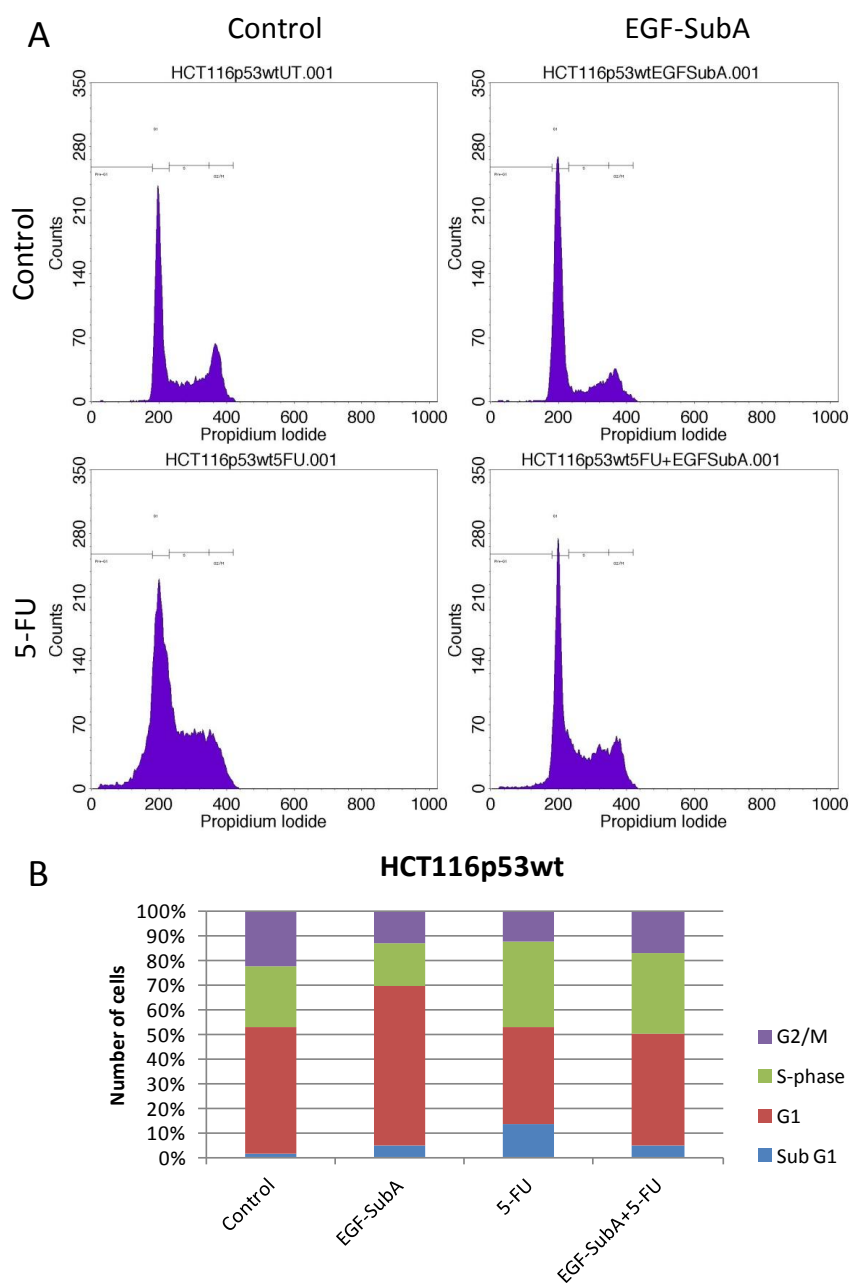


Figure 4.1.8-2: Cell cycle progression in HCT116p53wt cells treated with EGF-SubA and/or 5-FU.

HCT116p53wt cells were incubated with 2 nM EGF-SubA and/or 380 μ M 5-FU for 24 hr before harvest. The cells were stained with propidium iodide followed by flow cytometry analysis for cell cycle profile. (A) Representative cell cycle distribution of treated and untreated cells at 24 hr. (B) Histogram summarising cell cycle distribution from (A).

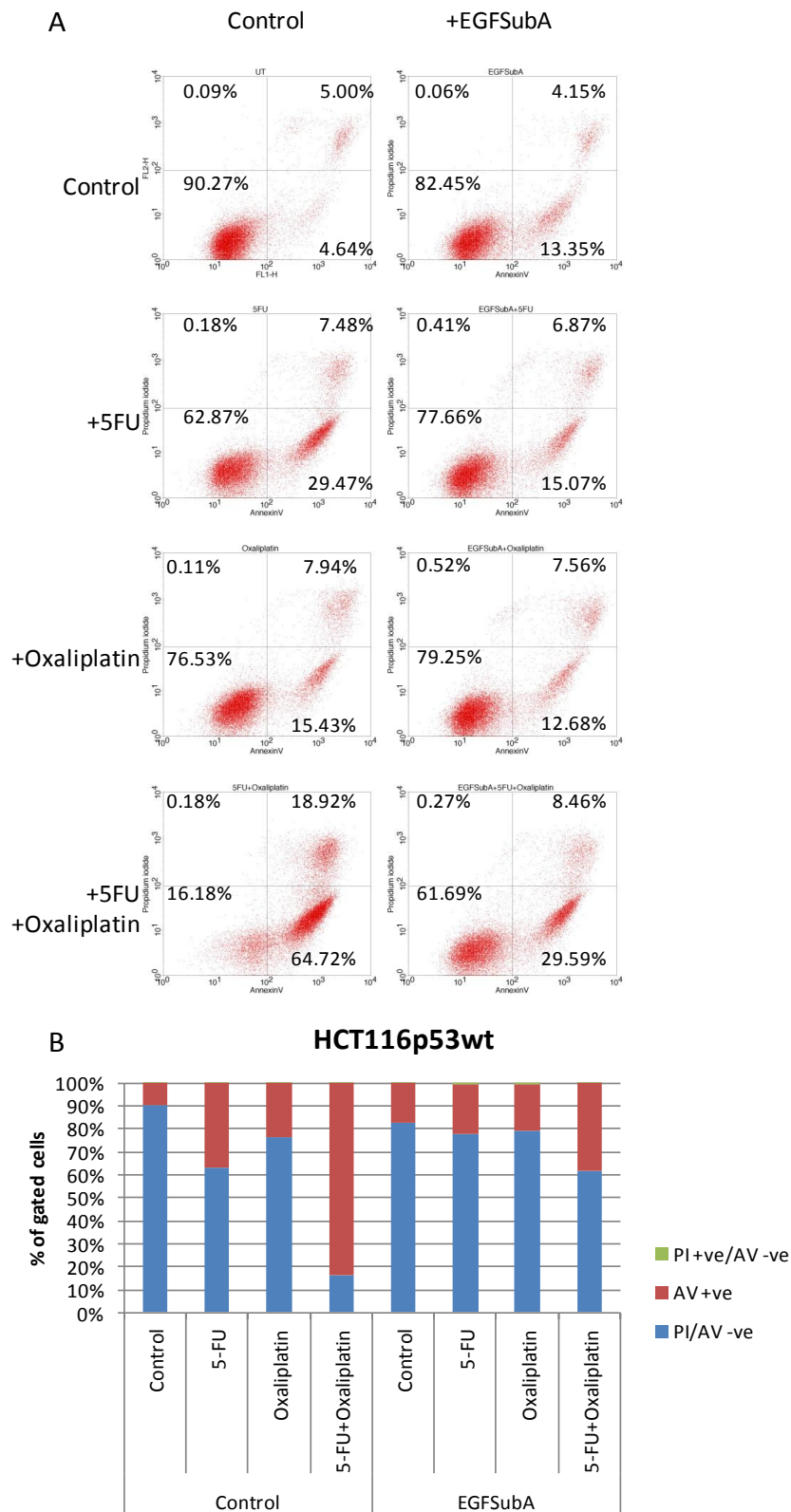


Figure 4.1.8-3: EGF-SubA reduces apoptotic response to 5-FU and Oxaliplatin in combination.

Experiment performed essentially as in the legend to Figure 4.1.8-1. Oxaliplatin (Hospira UK Ltd.) was added at a dose of 20 μ M.

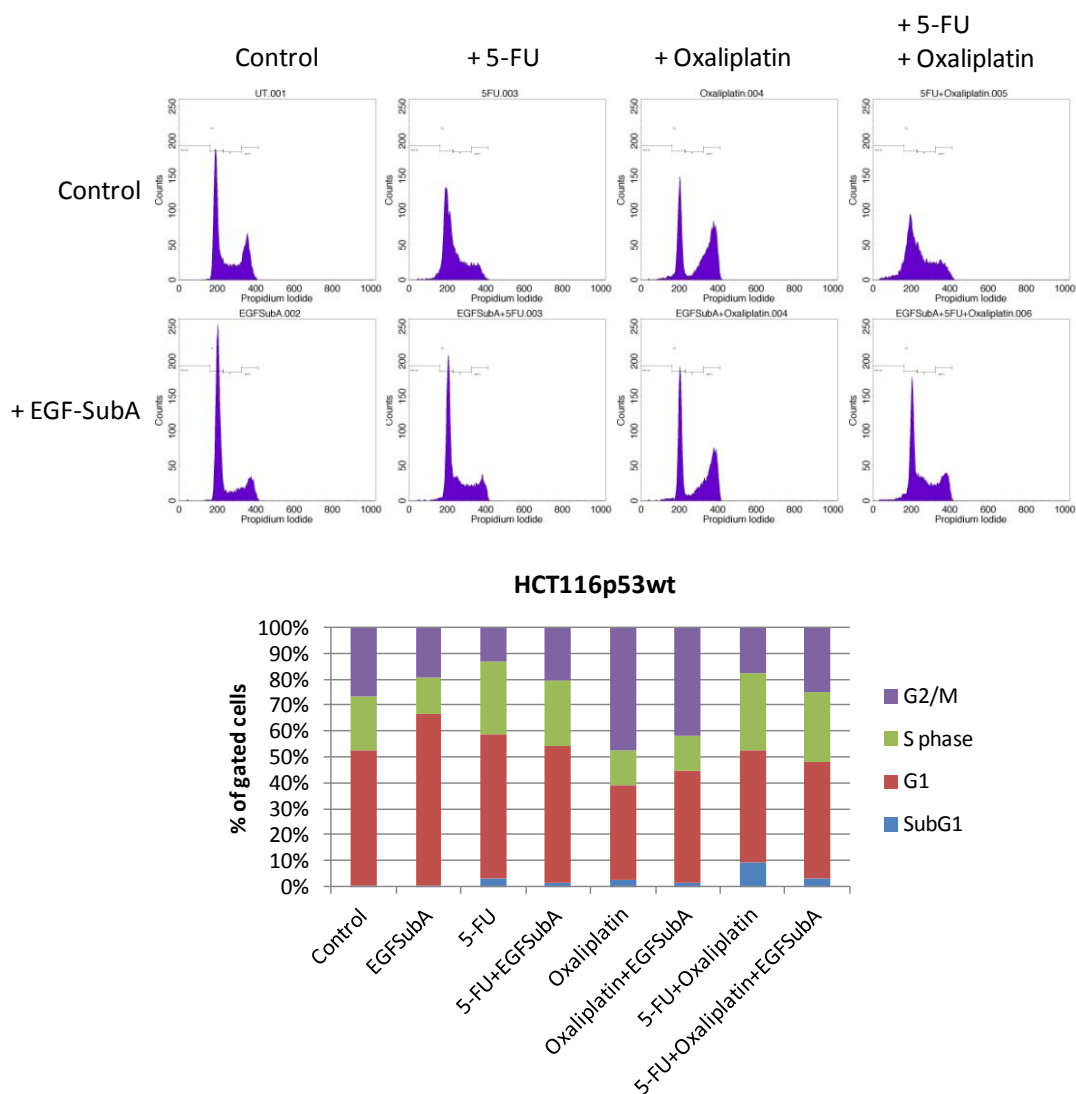


Figure 4.1.8-4: Cell cycle progression in HCT116p53wt cells treated with EGF-SubA and/or 5-FU and/or Oxaliplatin.

HCT116p53wt cells were incubated with 2 nM EGF-SubA and/or 380 μ M 5-FU and/or 20 μ M Oxaliplatin for 24 hr before harvest. The cells were stained with propidium iodide followed by flow cytometry analysis for cell cycle profile. (A) Representative cell cycle distribution of treated and untreated cells at 24 hr. (B) Histogram summarising cell cycle distribution from (A).

4.2 Discussion 2

Glucose-regulated protein 78 (GRP78), an endoplasmic reticulum (ER)-resident molecular chaperone, is essential for correct protein folding and assembly, and binds unfolded proteins so they can be repaired or targeted for proteosomal degradation. Micro-environmental stress and a requirement for increased protein synthesis, typical of solid tumours such as colorectal cancer, disrupts ER homeostasis causing accumulation of misfolded proteins. The ability of GRP78 to dissociate from several ER-resident transmembrane proteins during ER stress leads to a cascade of signal transduction pathways, known as the unfolded protein response (UPR), modulating cell survival or, if the stress is significantly severe, apoptosis.(202) The UPR allows cells to cope with an increase in unfolded proteins in the ER and may also prepare the cell for longer term adaptation through induction of chaperones such as GRP78 and modulation of the cells metabolic state.(229)

In the first section of this thesis, immunohistochemistry was used to confirm that GRP78 expression was increased in colorectal cancers compared with control normal mucosa. The proposed dependence upon GRP78 for cancer cell survival raises the potential of targeting this adaptive mechanism in cancer therapy.(202) Unlike other compounds which can reduce expression of GRP78, EGF-SubA causes highly specific cleavage at a single amino acid.(202, 314) This property led to the investigation of the therapeutic potential of targeting GRP78 in vitro. Using techniques including western blotting, MTT assay and flow cytometry, I have for the first time investigated the effects of EGF-SubA in colon cancer cells and also

assessed its combinatorial potential with contemporary drug treatments, namely 5-FU and oxaliplatin.

The weak apoptotic response to a 24hr oxaliplatin exposure may be regarded as a limitation in the above experiments, however the synergistic interaction with 5-FU can clearly be noted in the combination assays performed. Although cancer cells are highly sensitive to oxaliplatin in vitro, short exposure may only result in DNA-strand breaks and induction of apoptosis may require longer exposure to 48hr.(375, 376) Additionally, the above experiments did not explore the effect of a variable timing schedule in the delivery of 5-FU and oxaliplatin as both drugs were administered simultaneously. Any differential effects of timing schedule are likely to have been small however and previous data suggest that a synergistic interaction occurs whatever the tested combination schedule.(377) A more clinically relevant drug sequence consisting of Oxaliplatin for 2hr followed by 5-FU for 48 h (De Gramont regimen)(82) may have been a suitable option.

As reported for other cell lines,(314) colon cancer cells were highly sensitive to EGF-SubA at picomolar concentrations and sensitivity was a function of cell surface EGFR expression, with EGFR positive cells demonstrating a far greater reduction of proliferation in the presence of EGF-SubA than EGFR negative cells. EGF-SubA resulted in rapid cleavage of GRP78 and the evidence presented here suggests that this leads to activation of the UPR and increased expression of the chaperone GRP78, in keeping with the documented activity of subtilase cytotoxin (SubAB).(313) In addition to the induction of GRP78, increased transcription of the downstream UPR effector CHOP is also observed. In addition to its proapoptotic functions, CHOP is also known to mediate cell cycle arrest and analysis of

cytokinetics with flow cytometry confirmed that EGF-SubA causes a marked G1 arrest in EGFR-positive colon cancer cells.

In contrast to its potent effects upon proliferation, EGF-SubA was not found to be a strong inducer of apoptotic cell death, even when applied at high doses over several days. This raises the question of whether UPR activation by EGF-SubA might allow the cell to concentrate resources on mitigating stress rather than on growth and division, as indicated by the cell cycle arrest. Furthermore, when EGF-SubA was combined with 5-FU and oxaliplatin, the apoptotic response was significantly reduced, and this is likely to be due, at least in part, to cell cycle arrest. (Interestingly, elsewhere in this thesis it is shown that targeting GRP78 with siRNA, which had less effect upon the cell cycle, may also inhibit response to 5-FU and oxaliplatin, suggesting a possible wider role for GRP78 in the response to these drugs.)

Tumour cells are dependent upon pathways such as the UPR for survival. Is it possible that EGF-SubA, by way of its highly specific mode of action, is merely strengthening or enhancing the prosurvival response, rather than overwhelming or diminishing it? Through EGF-SubA mediated cleavage, simultaneous activation of PERK, IRE1 and ATF6 stress-signalling pathways occurs through dissociation from GRP78.⁽³¹³⁾ This in turn will lead to transcription of downstream UPR target genes. Although GRP78 is the master regulator of the UPR, removing it (via cleavage with EGF-SubA) does not impair the prosurvival effects of the UPR. The cell seems to respond to GRP78 cleavage in the same manner it would if GRP78 were to dissociate from its ER transmembrane sensors due to accumulation of misfolded protein in the ER.

Apoptosis resistance is a hallmark of malignancy. Tumour cells are able to overcome the apoptotic machinery and, hence, the propensity to be naturally eliminated. Conventional chemotherapy has limited effectiveness at least in part due to apoptosis resistance.(378) Cell cycle arrest will also lead to resistance to current cancer therapies that target actively dividing cells. The propensity for EGF-SubA to cause cell cycle arrest and resistance to apoptosis is likely to limit its potential as an anticancer therapy, particularly in the adjuvant setting where the aim is to eradicate micrometastatic deposits in patients who would otherwise be destined to have cancer recurrence. In the palliative setting, it could be envisaged that EGF-SubA might be able to halt progression of advanced disease and prolong survival but this is pure speculation.

Overall, the results in terms of the potential therapeutic value of targeting GRP78, are somewhat disappointing, especially given the optimism portrayed in previous reports of EGF-SubA.(314) In Backer's report, it was stated that EGF-SubA may synergize with ER stress-inducing drugs to kill cancer cells. In fact, what was actually demonstrated was that EGF-SubA inhibited proliferation (as determined by MTT assay) and this effect was increased in combination with thapsigargin. No evidence of 'killing' is actually presented and the authors actually comment that,

'...Interestingly, a 24-hour treatment with EGF-SubA alone did not result in any significant cleavage of α -fodrin or procaspase-7.'

In conclusion, despite its potency at inhibiting proliferation of tumour cells in vitro, the potential therapeutic value of EGF-SubA in the management of colorectal cancer is likely to be limited by its inhibition of apoptosis in combination with contemporary cytotoxic compounds, and the potential to activate prosurvival

mechanisms in colon cancer cells (which may in fact increase the likelihood of recurrence). Due to its highly specific cleavage of GRP78, EGF-SubA is certainly likely to remain useful as a research tool for investigating activation of the unfolded protein response.

5 Conclusion

Optimal surgery with complete resection of the tumour is the primary treatment for patients with resectable colorectal cancer. An additional survival benefit can be obtained from chemotherapy but selecting patients who are most likely to benefit from adjuvant therapy is complicated by lack of consistent data from biomarker studies, and thus in reality many patients are receiving treatment with a disadvantageous risk-benefit ratio. GRP78 has potential as a biomarker in that it is easily detectable; is well characterised as influencing tumour cell behaviour, drug sensitivity and malignant phenotype in vitro; is elevated in human tumours and less so in normal tissue, and expression is related to outcomes. Additionally, the role of GRP78 in tumorigenesis and adaptation to the hostile microenvironment found in solid tumours suggests that targeting GRP78 by small molecules may offer a potential therapeutic approach to cancer.

Using a validated antibody, GRP78 expression was characterised on a large cohort of surgically treated patients with colorectal cancer, many of whom received 5-FU based adjuvant chemotherapy. Tumours with low GRP78 expression were associated with worse survival and failure to benefit from chemotherapy. By targeting endogenous GRP78 using both siRNA and a novel engineered fusion protein, EGF-SubA, it was observed that in vitro, reduction of GRP78 reduced the efficacy of 5-FU through modulation of the cell cycle and apoptosis.

This exciting finding warrants further validation of GRP78 as a predictive biomarker through clinical research. The route to the use of such a biomarker in clinical practise though represents a considerable challenge. This may be achieved through retrospective or prospective methods. Due to the time and expense required to perform a prospectively designed randomised controlled trial (RCT), the possibility

to validate the predictive ability of GRP78 using data from a previously well-conducted RCT which compares adjuvant chemotherapy for which GRP78 is proposed to be predictive is a more feasible option. This would follow the CRUK Biomarker Roadmap with progression to the CTAAC/BIDD BM Qualification-Stage 1.(123) The essential elements required for a retrospective validation study are highlighted by Mandrekar and Sargent,(354) and are listed below:

1. Data from a well-conducted randomised controlled trial
2. Availability of samples on a large majority of patients to avoid selection bias
3. Prospectively stated hypothesis, analysis techniques, and patient population
4. Predefined and standardized assay and scoring system
5. Upfront sample size and power justification

An example of a suitable RCT to validate GRP78 would be the QUASAR trial.(83) This was a multicentre international RCT enrolling over 3000 patients and conducted during the same period of time the Liverpool cohort was recruited, and patients received comparable adjuvant chemotherapy regimens. Tissue from trial patients is held on tissue microarray and would be suitable for immunohistochemistry, thus, the reproducibility and accuracy of GRP78 analysis by this method could be established. Such an analysis would potentially provide compelling evidence for GRP78 as a potential predictive tool to select patients who may or may not benefit from fluorouracil-based chemotherapy.

The gold standard for validation of a predictive biomarker is a prospective RCT (CRUK CTAAC/BIDD BM Qualification-Stage2,(123)). Providing that retrospective analyses confirmed its potential as a predictive biomarker then a GRP78 stratified trial could be designed.(379) Rather than restrict eligibility to a trial, stage 3 patients

could be stratified using a priori cut-off points into GRP78 high and GRP78 low, then randomly assigned to either standard therapy (FOLFOX) or an experimental group receiving, for example, FOLFOX plus a new treatment such as Bevacizumab (Figure 4.1.8-1).

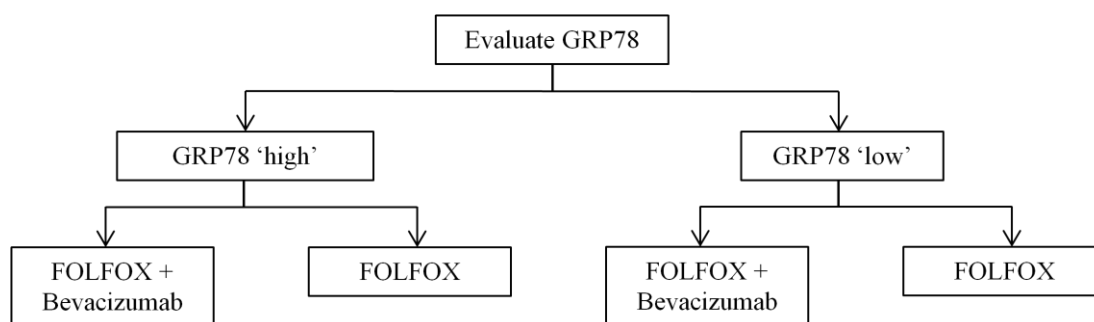


Figure 4.1.8-1: Marker-by-treatment-interaction design for a prospective predictive marker validation.

This study design is similar to conducting two independent RCT's, one in each GRP78-based subgroup. One may envisage that the GRP78 low group may receive the most potential benefit of addition of Bevacizumab over FOLFOX alone.

In-vitro, EGF-SubA mediated GRP78 cleavage was toxic to colon cancer cells with sensitivity governed by EGFR expression at the cell surface. Cleavage of GRP78 resulted in activation of the UPR and cell cycle arrest but EGF-SubA was not a potent inducer of apoptosis and combinations of EGF-SubA with 5-FU and Oxaliplatin were actually antagonistic. These findings suggest that despite targeting a prosurvival mechanism, EGF-SubA is not likely to have a therapeutic role in colorectal cancer management. However, due to its highly specific cleavage of GRP78, EGF-SubA is of value as a scientific tool with which to study GRP78 and the UPR in vitro. Its role in other tumour types is yet to be determined.

Together, the findings of this research demand a re-evaluation of our understanding of the role of GRP78, a well characterised mediator of drug resistance in cancers. I

propose that cells lacking GRP78 are more likely to activate the UPR, undergo cell cycle arrest and therefore exhibit drug resistance, whereas cells with abundant GRP78 are successfully adapted to their microenvironment and by their proliferative state, are more sensitive to cytotoxic agents such as 5-FU. This poses the question of in which situation is a patient better off; to have a slow growing tumour that exhibits drug resistance, or a tumour freed from the confines of its microenvironment that can grow but is more sensitive to chemotherapy?

6 Appendix

6.1 Commonly used chemotherapy regimens in colorectal cancer

Table 6.1-1: Commonly used chemotherapy regimens in colorectal cancer.

Regimens Used in the Treatment of Colon Cancer	
Regimen	Drugs and Schedule
Mayo FU/LV	Days 1-5: fluorouracil 425 mg/m ² IV bolus + leucovorin 20 mg/m ² IV bolus Repeat every 4 weeks.
LV5FU2	Days 1 and 2: leucovorin 200 mg/m ² IV bolus + 400 mg/m ² fluorouracil IV bolus + 600 mg/m ² CIVI over 22 hours Repeat every 2 weeks for 12 cycles.
FULV	Day 1: fluorouracil 500 mg/m ² IV bolus + leucovorin 500 mg/m ² IV bolus Repeat weekly for 6 weeks, then rest 2 weeks and repeat.
Capecitabine	Capecitabine 1,250 mg/m ² po twice daily
IFL	Day 1: fluorouracil 500 mg/m ² + leucovorin 20 mg/m ² + irinotecan 125 mg/m ² Repeat weekly for 6 weeks.
FOLFIRI	Day 1: irinotecan 180 mg/m ² + leucovorin 100 mg/m ² IV + fluorouracil 400 mg/m ² IV bolus, then fluorouracil 600 mg/m ² CIVI Day 2: leucovorin 100 mg/m ² IV bolus + fluorouracil 400 mg/m ² IV bolus, then fluorouracil 600 mg/m ² CIVI Repeat every 2 weeks.
FLOX	Day 1: fluorouracil 500 mg/m ² IV bolus + leucovorin 500 mg/m ² IV bolus + oxaliplatin 85 mg/m ² IV Repeat fluorouracil and leucovorin weekly for 6 weeks then rest 2 weeks and repeat the 8-week cycle. Repeat oxaliplatin on weeks 1, 3, and 5 of each 8-week cycle.
FOLFOX	Day 1: leucovorin 200 mg/m ² IV bolus + fluorouracil 400 mg/m ² IV bolus + fluorouracil 600 mg/m ² CIVI over 22 hours + oxaliplatin 85 mg/m ² IV Day 2: leucovorin 200 mg/m ² IV bolus + fluorouracil 400 mg/m ² IV bolus + fluorouracil 600 mg/m ² CIVI over 22 hours Repeat days 1 and 2 every 2 weeks until progression or intolerance.
FOLFOX4	Day 1: leucovorin 200 mg/m ² IV bolus + fluorouracil 400 mg/m ² IV bolus + fluorouracil 600 mg/m ² CIVI over 22 hours + oxaliplatin 85 mg/m ² IV Day 2: leucovorin 200 mg/m ² IV bolus + fluorouracil 400 mg/m ² IV bolus + fluorouracil 600 mg/m ² CIVI over 22 hours Repeat days 1 and 2 every 2 weeks for 12 cycles.
IROX	Day 1: irinotecan 200 mg/m ² IV + oxaliplatin 85 mg/m ² IV Repeat every 3 weeks.
CapeOx	Day 1: oxaliplatin 130 mg/m ² IV + capecitabine 1,000 mg/m ² po twice daily for 14 days Repeat every 3 weeks.
FULV with bevacizumab	Day 1: fluorouracil 500 mg/m ² IV bolus + leucovorin 500 mg/m ² IV bolus + bevacizumab 5 mg/kg or 10 mg/kg IV Repeat FULV weekly for 6 weeks, then rest 2 weeks and repeat. Repeat bevacizumab every 2 weeks until disease progression or intolerance.
Cetuximab	Week 1: cetuximab 400 mg/m ² IV Subsequent doses cetuximab 250 mg/m ² IV repeated weekly
Cetuximab with irinotecan	Week 1: cetuximab 400 mg/m ² IV + irinotecan (various doses) Subsequent doses cetuximab 250 mg/m ² IV repeated weekly
Cetuximab with FOLFOX	Day 1: leucovorin 200 mg/m ² IV bolus + fluorouracil 400 mg/m ² IV bolus + fluorouracil 600 mg/m ² CIVI over 22 hours + oxaliplatin 85 mg/m ² + cetuximab 400 mg/m ² IV Day 2: leucovorin 200 mg/m ² IV bolus + fluorouracil 400 mg/m ² IV bolus + fluorouracil 600 mg/m ² CIVI over 22 hours Repeat days 1 and 2 of FOLFOX every 2 weeks until progression or intolerance. Subsequent doses of cetuximab 250 mg/m ² IV repeated weekly.
Panitumumab	Panitumumab 6 mg/kg IV Repeat every 2 weeks.

CIVI: continuous intravenous infusion.

6.2 Flow cytometer setting for bivariate Annexin V/Propidium iodide analysis

Cytometer Type: FACSCalibur

Detectors/Amps:

Param	Detector	Voltage	AmpGain	Mode
P1	FSC	E-1	5.52	Lin
P2	SSC	380	1.00	Lin
P3	FL1	417	1.00	Log
P4	FL2	462	1.00	Log
P5	FL3	809	1.00	Log
P6	FL2-A		1.00	Lin
P7	FL2-W		1.00	Lin

Threshold:

Primary Parameter: FSC
Value: 52

Secondary Parameter: None

Compensation:

FL1 - 0.0 % FL2
FL2 - 72.4 % FL1
FL2 - 0.0 % FL3
FL3 - 0.0 % FL2

6.3 Targeting of GRP78 using a different siRNA

6.3.1 siRNA targeting GRP78, siGENOME D-008198-05

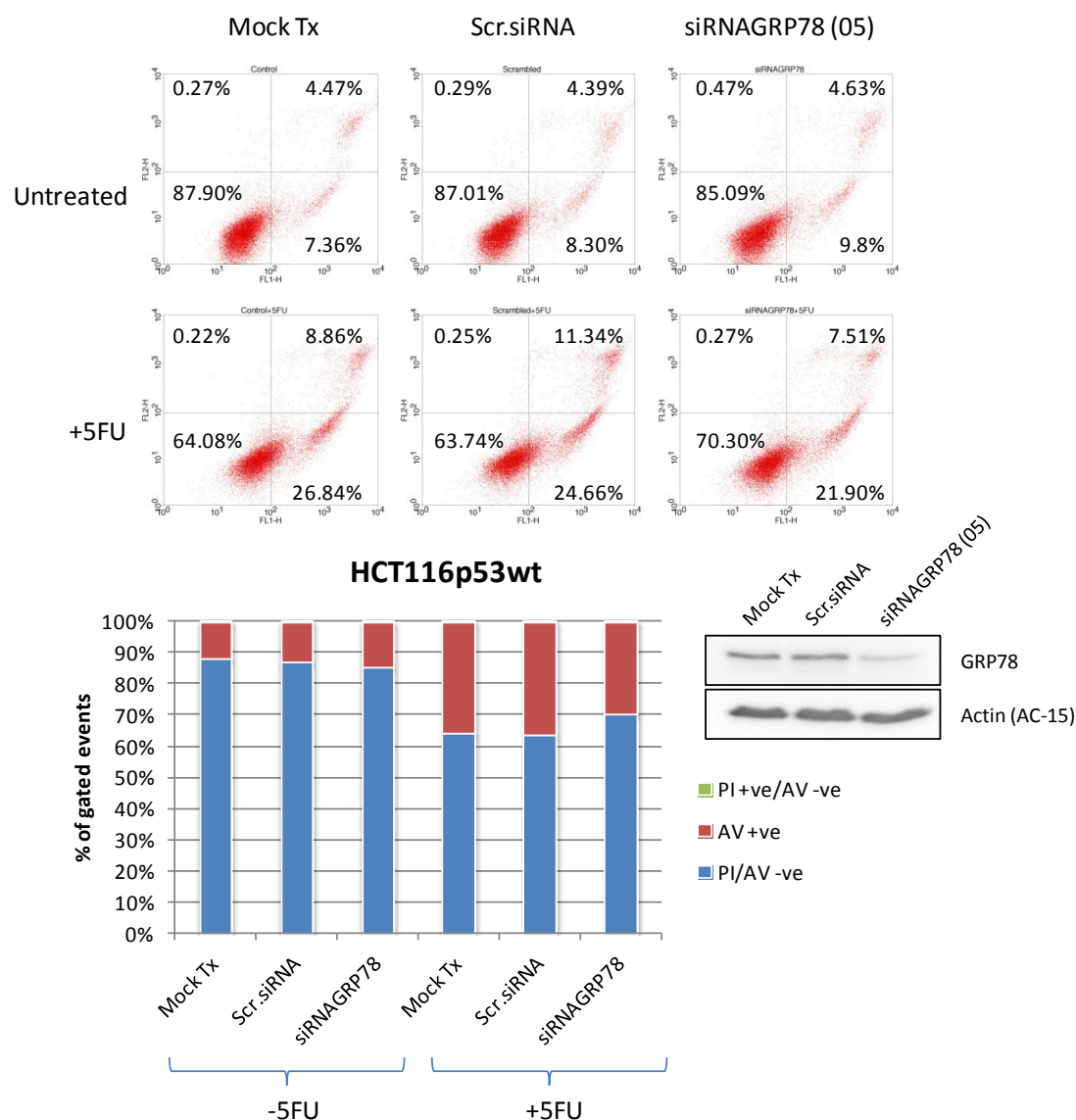


Figure 6.3.1-1: Knockdown of GRP78 with a different siRNA produces similar reduction of apoptosis.

The experiment performed as per Figure 3.1.11-1 but using a different GRP78 targeting siRNA (siGENOME D-008198-05). Cells were seeded 24 hours prior to transfection with a final siRNA concentration of 40 nM. A mock transfection control was prepared identically except for omitting the siRNA oligonucleotide. 48 hr after transfection, cells were treated +/- 100 μ M 5-FU (medac GmbH, Germany) for 24 hr then harvested for flow cytometry.

6.4 Difficulties of modulating GRP78 and assessing chemosensitivity in-vitro

6.4.1 Overview

During the work performed for this thesis, some of the challenges of trying to model the UPR and manipulate GRP78 in vitro became increasingly apparent.

The induction of GRP78 is well established as a marker for ER stress.(225) A number of researchers have used low glucose stress as a means of upregulating GRP78 expression and modelling the effects of the UPR.(276, 296) Indeed, GRP78 was first characterised because of its induction by glucose deprivation.(192) Others have induced ER stress and upregulation of GRP78 by treating cells with the NAD antagonist 6-aminonicotinamide (6AN) or inhibitors of glucose metabolism such as 2-deoxyglucose (2dG).(288, 292) The use of the glycosylation inhibitor tunicamycin,(265) and depletion of intracellular calcium using calcium ionophores or thapsigargin are also well documented.(193, 200)

As a starting point, I investigated the effects of glucose starvation in colon cancer cells. Culture under conditions of progressive glucose deprivation for 24 hours induced expression of GRP78 on western blotting (Figure 6.4.1-1). This effect did not appear to be short lived as further investigation demonstrated that GRP78 remained elevated at 96 hr of culture in low glucose (Figure 6.4.1-2).

Having demonstrated that low glucose stress can induce expression of GRP78 in vitro, I initially assumed that this model could be used to study the effects of modulating GRP78 upon drug sensitivity. As an example, I used the MTT assay in several experiments to assess the response to treatment with oxaliplatin under

conditions of low glucose stress known to induce GRP78 expression. The results of these experiments demonstrate that culture of cells in low glucose media (that will cause ER stress and induce GRP78 expression) can cause resistance to oxaliplatin as deduced by the increased survival compared with the control (Figure 6.4.1-3). The conclusion may be drawn that the drug resistance is due to elevated GRP78.

However, this experiment and the conclusions drawn from it are flawed as shall be demonstrated. If this experiment is repeated to include analysis of the proliferation of cells under each of the conditions, the reasons behind the flaw become clear. Although the data represented by histograms of relative percentage survival indicates increased survival in low glucose following oxaliplatin treatment, the proliferation curves demonstrate that this effect is due to a marked reduction in proliferation in the cells exposed to 1 mM glucose (Figure 6.4.1-4). As these cells are barely proliferating, any additional inhibition due to drug treatment appears to account for a relatively smaller percent than those cultured in standard growth media. Therefore it is incorrect to draw the conclusion that it is specifically the elevated GRP78 responsible for drug resistance. This conclusion has been drawn in published reports using this technique. I found two papers, one in the British Journal of Cancer and the other in the European Journal of Gastroenterology & Hepatology, which use this technique to conclude that increased GRP78 is responsible for resistance to doxorubicin and 5-FU in breast and hepatocellular carcinoma.(296, 380) Both papers have been cited a number of times. One should in fact conclude that it is UPR activation and reduced proliferation leading to drug resistance, and that elevated levels of GRP78 are only a marker for ER stress and not causative. This experiment

has not tested specific modulation of GRP78 as the conditions used have other effects upon proliferation.

Analysis of alterations in chemosensitivity upon manipulation of GRP78 may be best performed using an assay that is not so reliant upon the effects of cell proliferation, such as flow cytometry. It is interesting to compare the results of an Annexin V assay with those of the MTT assay when used to assess response to 5-FU in cells cultured in low glucose conditions. Figure 6.4.1-5 demonstrates that cells cultured in 2 mM glucose have a significant reduction in apoptosis compared with cells in standard media when exposed to 5-FU, and as Figure 6.4.1-6 reveals, this is largely due to cell cycle arrest caused by low glucose stress. Therefore, the conclusion is that cell stress, brought about by culture in low glucose medium, leads to cell cycle arrest and reduced sensitivity to 5-FU. It is not elevated GRP78 independently that is causing resistance.

Similarly, when I performed a combination study of EGF-SubA and 5-FU (as described in section 4.1.7), isobolograms constructed using data from MTT assay suggested an additive, if not synergistic, effect of combining these drugs. However, subsequent experiments using the Annexin V assay revealed that EGF-SubA significantly reduces the apoptotic effects of 5-FU (see Figure 4.1.8-1). The paradox is that EGF-SubA, known to cause highly specific cleavage of GRP78, can cause additional growth inhibition when combined with 5-FU, but at the same time is inhibiting the apoptotic effects. EGF-SubA, due to its specificity, is a useful tool for studying GRP78 in vitro but in addition to its rapid and specific depletion of intracellular GRP78, the SubA component is also a major activator of the UPR and can cause cell cycle arrest.(313, 369) Cell cycle arrest in G1 was confirmed in my

experiments and is a possible explanation for the reduced efficacy of 5-FU (see Figure 4.1.5-3 and Figure 4.1.8-2).

The difficulty that presented itself was that the above methods of modulating GRP78 led to activation of the UPR which might otherwise confound interpretation through the pleiotropic effects of the stress. It is also possible that such severe, acute forms of stress do not accurately model conditions in vivo and may be resulting in non-physiological levels of GRP78. These issues led me to investigate the use of GRP78 knockdown using siRNA. The technique benefits from the fact that it is modulating physiologically relevant levels of endogenous GRP78. Although western blots confirm good reduction of the levels of GRP78, the effect on the cell cycle was minimal, in contrast to the effects seen with EGF-SubA or glucose starvation (Figure 6.4.1-6).

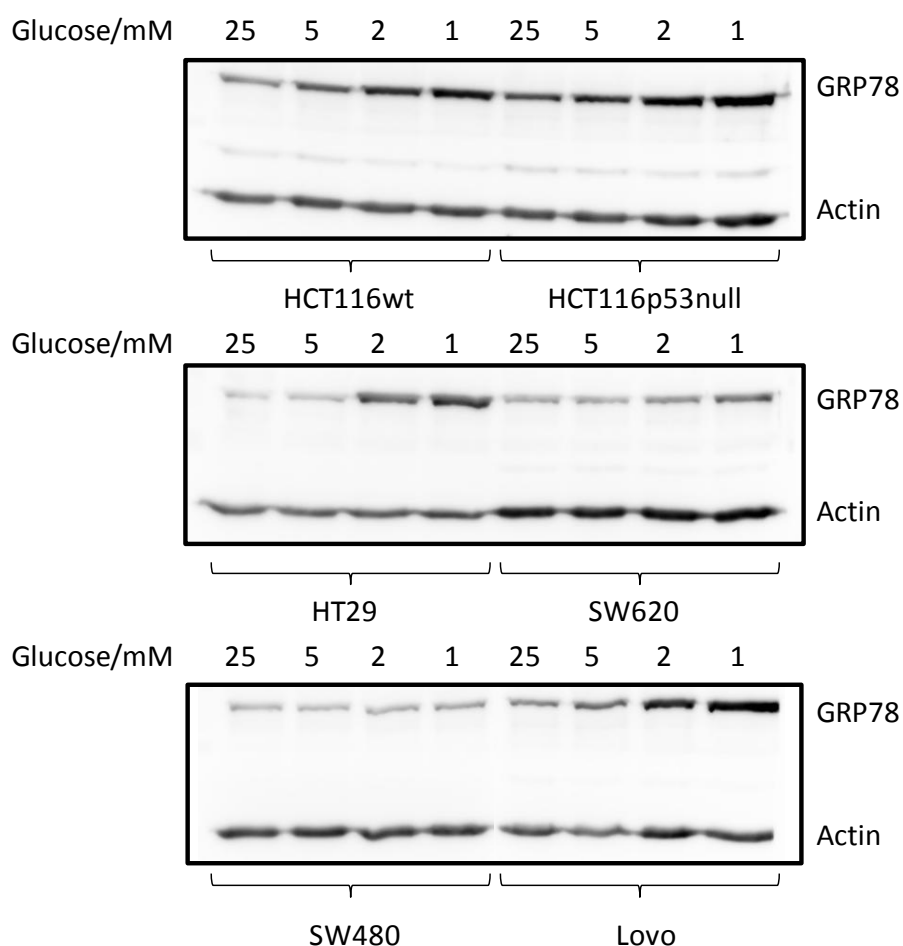


Figure 6.4.1-1: Western blots of colon cancer cell line lysates after exposure to culture conditions with varying glucose concentrations to demonstrate GRP78 expression.

Standard Dulbecco's Modified Eagle's Medium (DMEM) HEPES Modified (Sigma) containing 25 mM glucose (Sigma) was combined with glucose free DMEM (Gibco) to give a range of glucose concentrations. Cells cultured in 6 well plates were harvested after 24 hr of exposure to different glucose concentrations and cell lysates were subjected to western blot. Actin was used as a loading control.

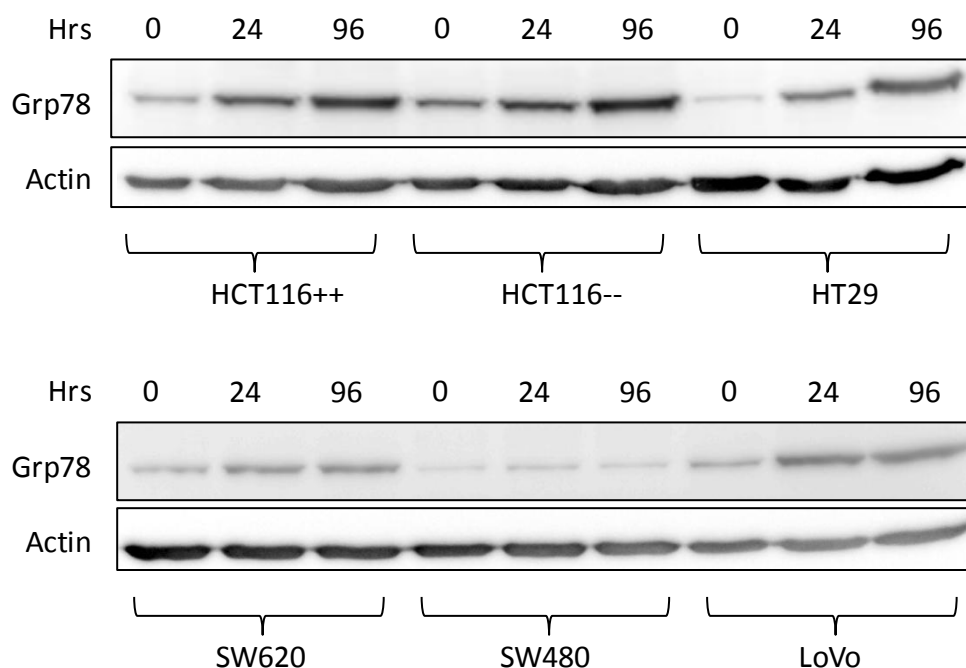


Figure 6.4.1-2: Western blot demonstrating that GRP78 remains elevated at 96 hours of low glucose stress.

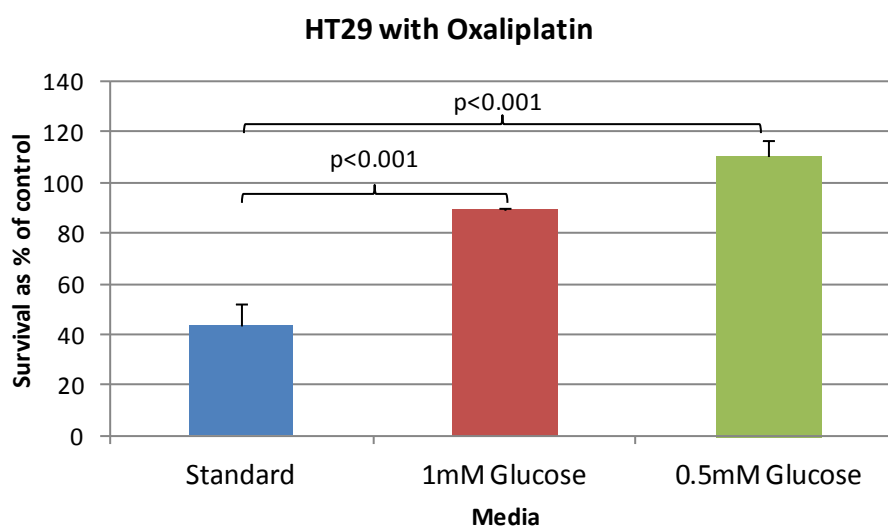


Figure 6.4.1-3: Relative % toxicity for HT29 cells under normal and glucose-deprived culture conditions when exposed to previously determined IC₅₀ doses of Oxaliplatin.

Relative % survival (mean \pm s.e.m.) for HT29 cells under normal and glucose-deprived culture conditions when exposed to 0.3 μ M Oxaliplatin for 72 hr. Viability of control untreated cells was defined as 100%. Survival appears significantly higher under conditions of low glucose compared with standard culture conditions. Data derived from quadruplicate repeats, P values calculated using student t-test.

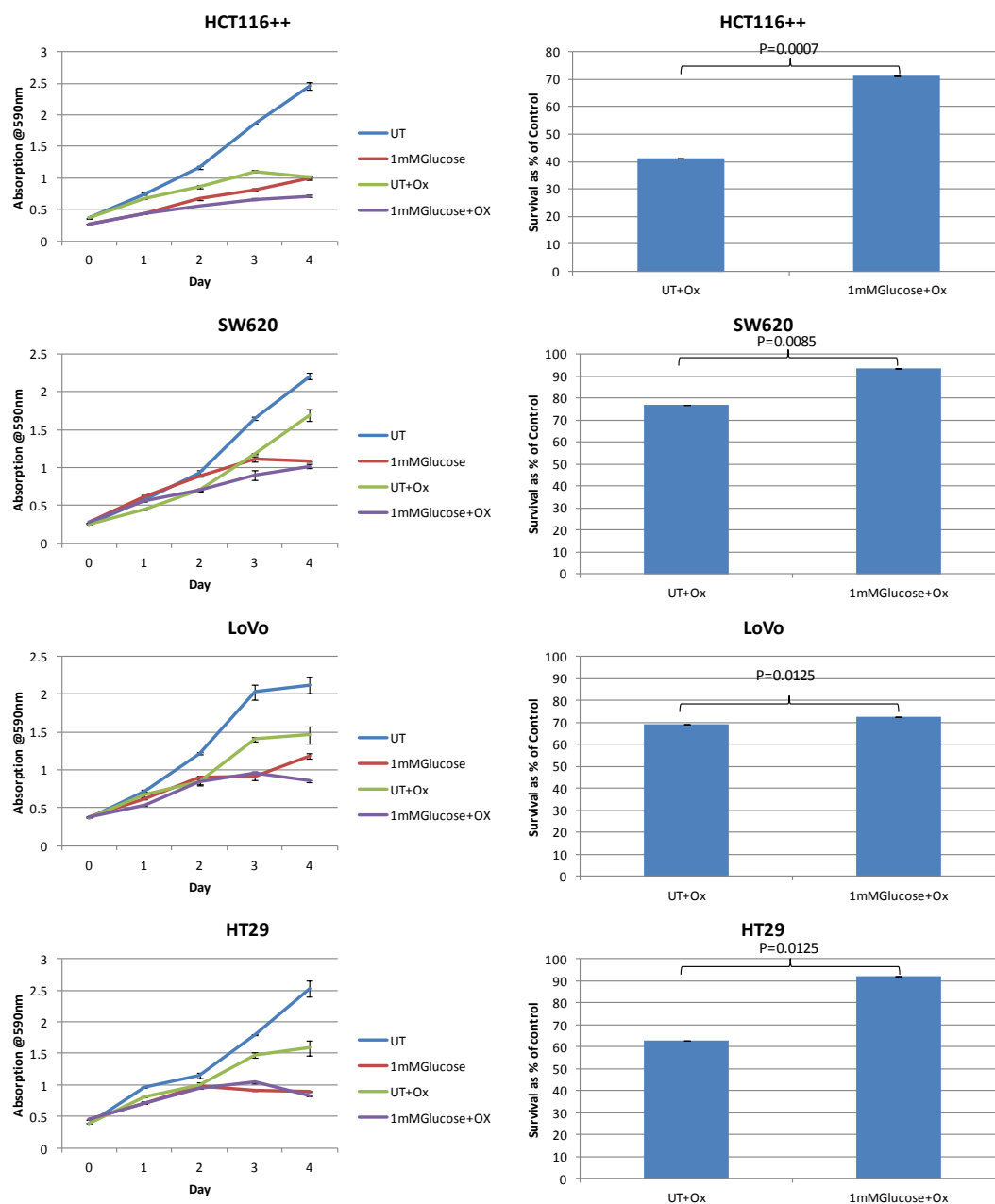


Figure 6.4.1-4: Higher survival after oxaliplatin treatment is a function of reduced proliferation under low glucose culture rather than reduced sensitivity.

Colon cancer cells were seeded to 96 well plates and allowed to settle for 24 hr after which fresh standard growth media or media containing 1 mM glucose supplemented with a predetermined IC_{50} dose of oxaliplatin or vehicle control was added. MTT assay was performed daily to chart proliferation. Media and drugs were changed daily. A histogram representing relative % survival (mean + s.e.m.) at day 4 is presented for each cell line. Viability of control untreated cells was defined as 100%. Data derived from quadruplicate repeats, P values calculated using student t-test.

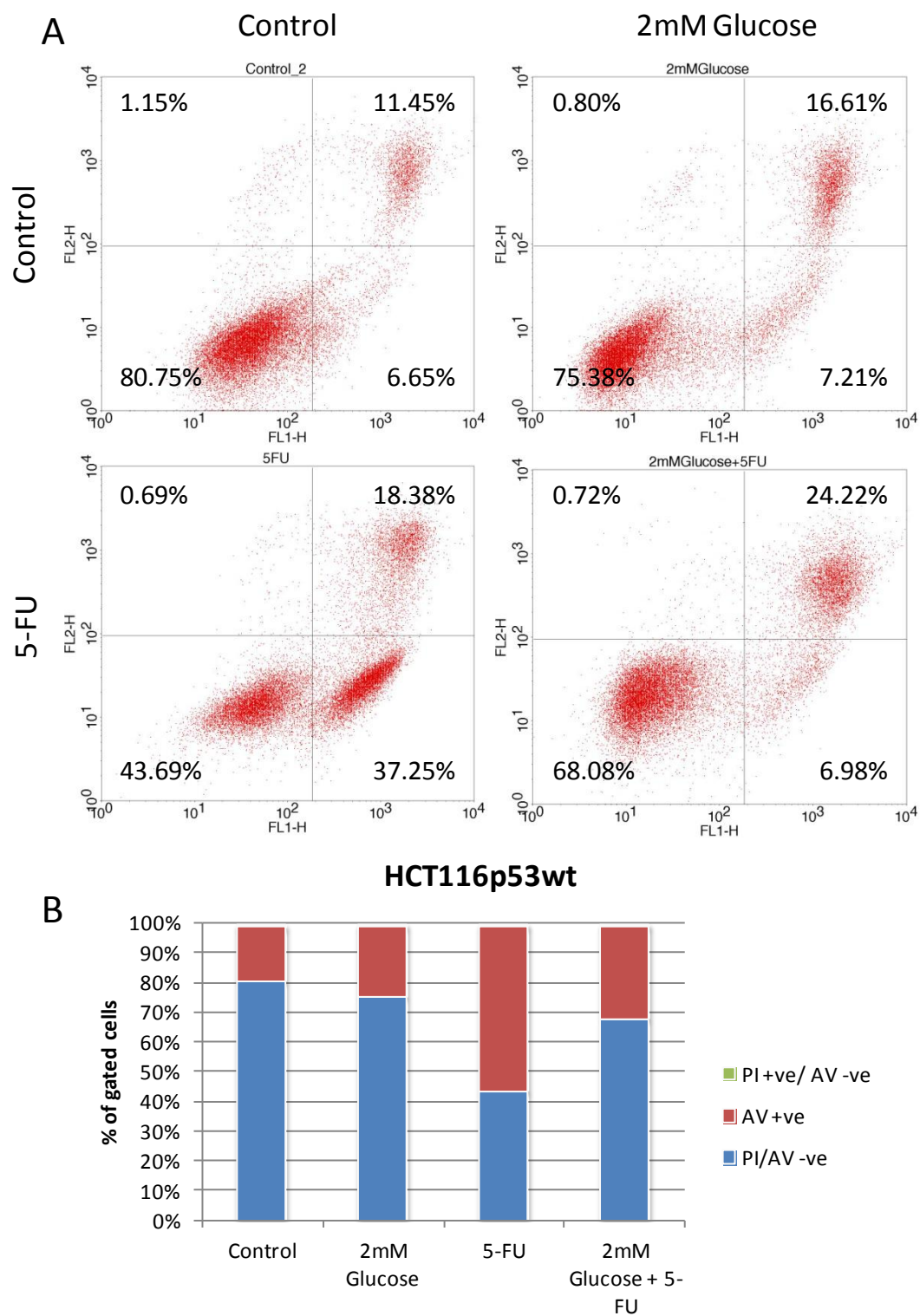


Figure 6.4.1-5: 5-FU induced apoptosis is reduced in cells cultured in low glucose media.

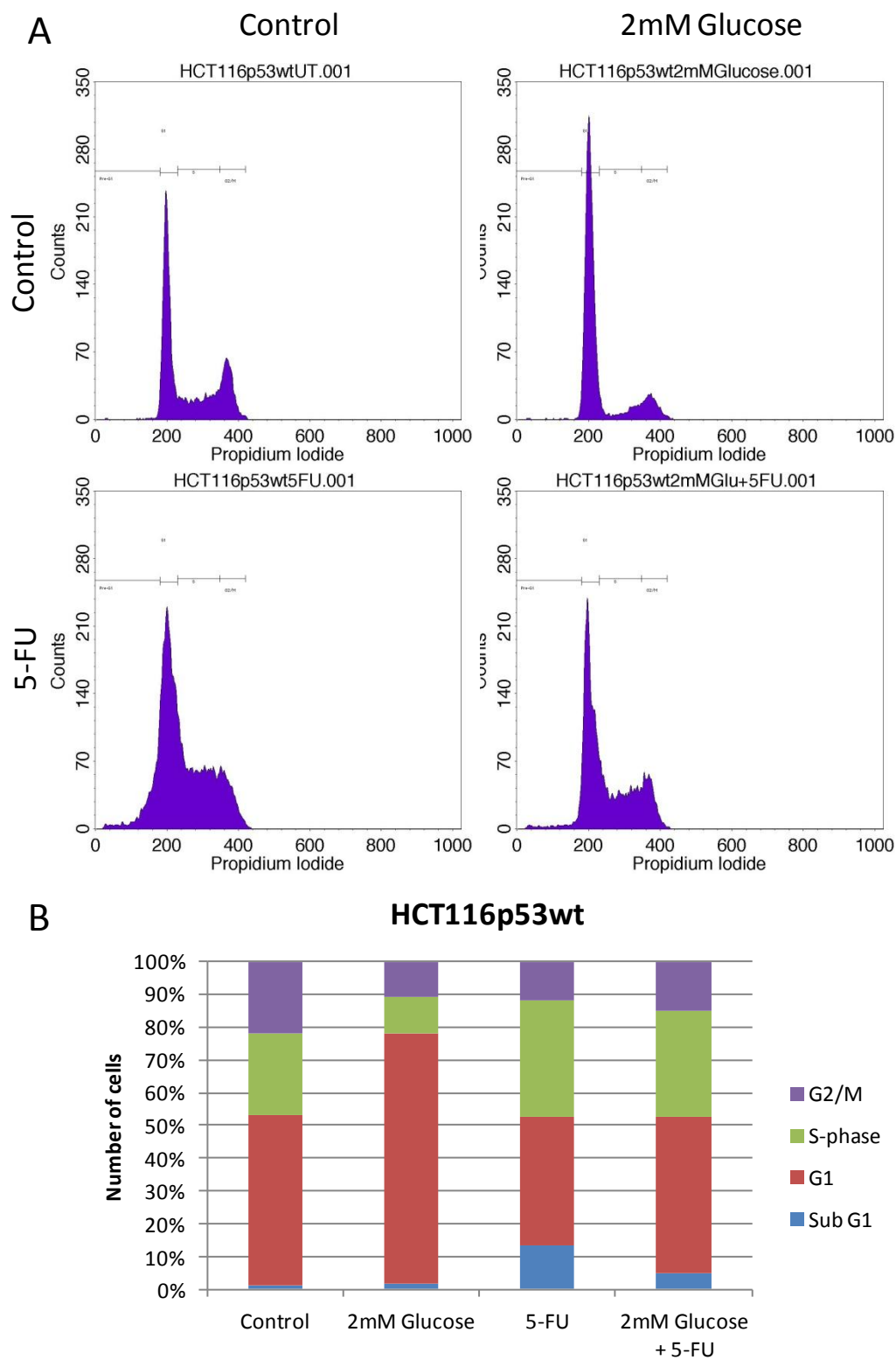


Figure 6.4.1-6: Glucose starvation of colon cancer cells in vitro leads to cell cycle arrest in G1 and abrogates the effects of 5-FU upon the cell cycle.

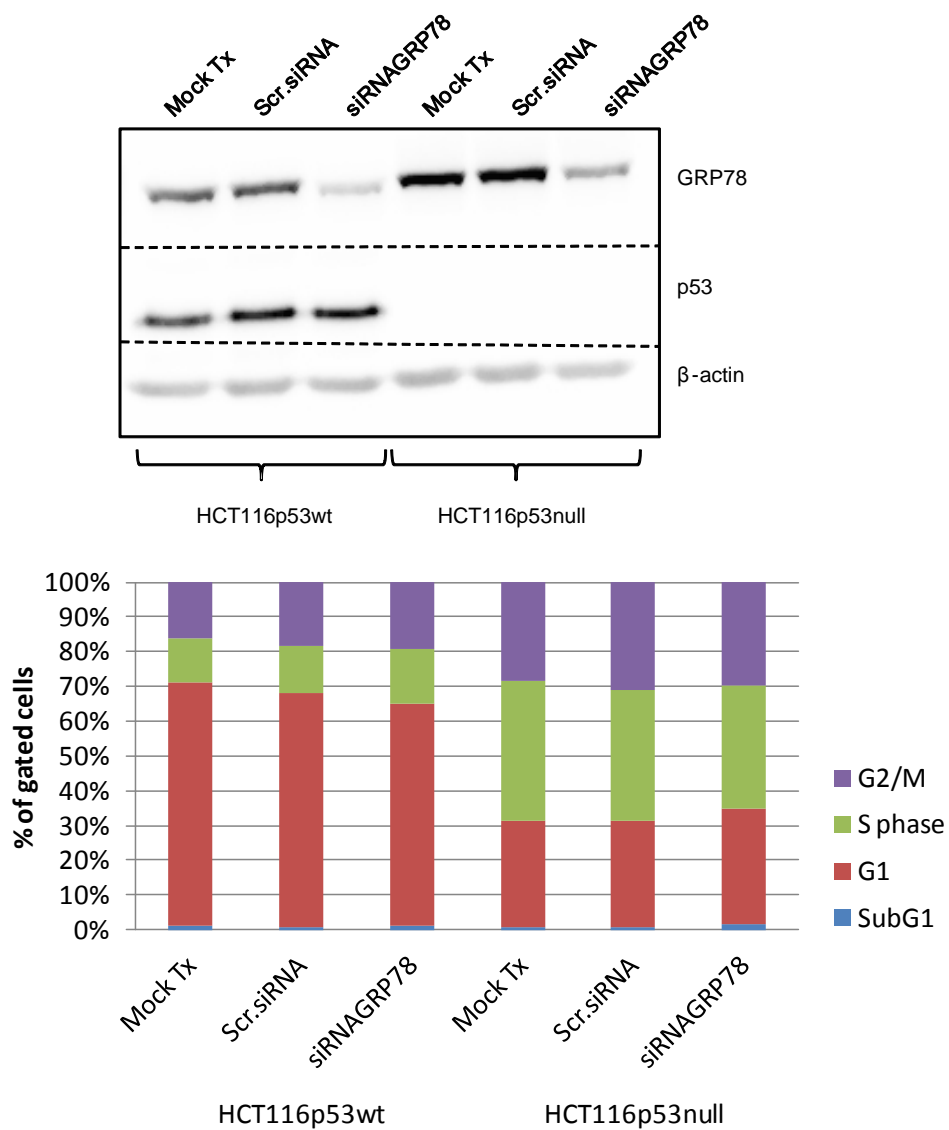


Figure 6.4.1-7: Targeting GRP78 with siRNA has minimal effect upon the cell cycle.

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